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**THE CONTROL OF HUMAN CYTOMEGALOVIRUS LATENT AND LYTIC
GENE EXPRESSION BY CHROMATIN REMODELLING**

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It is now well established that the differentiation-dependent control of human cytomegalovirus (HCMV) latent infection is regulated by the association of chromatin around the major immediate early promoter (MIEP). However, whether chromatin-mediated regulation of viral immediate early (IE) gene expression also occurs during a productive infection is not known. Consequently, the role of chromatin structure on viral gene expression during HCMV infection of a permissive cell type was investigated.

Experiments demonstrated that the MIEP was, indeed, associated with chromatin throughout infection and was subject to modification consistent with the known temporal transcription of IE genes. However, immediately upon infection, the MIEP was associated with a repressed chromatin structure. This repression was relieved through use of the histone deacetylase (HDAC) inhibitor Trichostatin-A (TSA), confirming that the regulation of IE gene expression at this time was mediated by an inhibitory chromatin structure. Furthermore, down-regulation of the ND10-associated transcriptional repressor, hDaxx, which is known to interact with HDACs, led to similar relief from repression. Further analysis of promoters of prototypic viral early and late genes, UL44 and pp28 respectively, also revealed that all classes of viral genes are subject to chromatin-mediated regulation throughout productive infection, consistent with their known transcription profile.

Although differentiation-dependent regulation of MIEP activity underpins viral latency and reactivation, down-regulation of the intrinsic anti-viral repressor hDaxx in *in vitro* latent model systems did not permit reactivation of IE gene expression. Consequently, in contrast to lytic viral infection, repression of IE gene expression by hDaxx does not appear to be involved in regulation of viral latency and reactivation.

In conclusion, immediately upon infection of permissive cells, intrinsic repression of HCMV IE gene transcription occurs through the establishment of an inhibitory chromatin structure around the MIEP, mediated by hDaxx and HDACs. This repression is overcome by viral factors before full productive infection can begin. Full lytic infection then involves regulation of viral gene expression through remodelling of chromatin structure at all classes of HCMV promoters.

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work performed by others. The work was performed whilst I was a graduate student at the Department of Medicine, University of Cambridge under the supervision of Prof. John Sinclair.

A handwritten signature in dark ink, appearing to be 'I. Groves', with a long horizontal stroke extending to the right.

Ian Groves (16-May-2008)

The length of this thesis does not exceed the word limit set by the Clinical Medicine and Clinical Veterinary Medicine Degree Committee.

Abbreviations

A	Adensoine
AAV	Adeno-associated virus
ac	Acetylated
AIDS	Acquired immunodeficiency syndrome
AMV	Avian myeloblastosis virus
AP	Assembly protein
APL	Acute promyelocytic leukaemia
ATCC	American tissue culture collection
ATP	Adensoine triphosphate
ATRX	α -thalassaemia/mental retardation syndrome X-linked
AuBK	Aurora B kinase
BCR	B cell receptor
bp	Base pair
BPV	Bovine papillomavirus
C	Cytosine
cAMP	Cyclic adenosine monophosphate
cat	Chloramphenicol acetyltransferase
CBP	CREB binding protein
CBX1	Chromobox homolog 1 (HP1 β)
CD	Cluster of differentiation
cdk	Cyclin-dependent kinase
Cdt1	Chromatin licensing and DNA replication factor 1
CDV	Cidofovir
C/EBP	CCAAT-enhancer binding protein
ChIP	Chromatin immunoprecipitation
CK2	Casein kinase 2
CLT	Cytomegalovirus latency-specific transcript
CMV	Cytomegalovirus
CO₂	Carbon dioxide
CPE	Cytopathic effect
CR2	Complement receptor 2
CRE	cAMP response-element
CREB	cAMP response-element binding protein
crs	Cis repression sequence
Ct	Cycle threshold
CTL	Cytotoxic T lymphocyte
Daxx	Death domain-associated protein 6

DB	Dense body
DC	Dendritic cell
DE	Delayed early
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dsDNA	Double stranded DNA
E	Early
EBER	Epstein-Barr encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EC	Endothelial cell
ECACC	European Collection of Cell Cultures
EGF	Epidermal growth factor
EGF-R	EGF-receptor
EIHV-1	Endotheliotropic elephant herpesvirus-1
EM	Electron microscopy
ERF	Ets-2 repressor factor
ES	Embryonic stem [cell]
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration (US)
FITC	Fluorescein isothiocyanate
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gB/H/L/O	Glycoprotein B/H/L/O
GCV	Ganciclovir
Gfi-1	Growth factor independence-1
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GMP	Granulocyte-macrophage precursor
h	Human
H	Histone
HAT	Histone acetyltransferase
HAUSP	Herpesvirus-associated ubiquitin-specific protease (USP7)
HC	Hydrocortisone
HCV	Hepatitis C virus
HCMV	Human CMV
HDAC	Histone deacetylase
HFF	Human foreskin fibroblast
HFL	Human foetal lung cell

HFV	Human foamy virus
HHV	Human herpesvirus
HIPK1	Homeodomain-interacting protein kinase 1
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMT	Histone methyltransferase
HP1	Heterochromatin protein 1
HPV	Human papillomavirus
HSV	Herpes simplex virus
ICP0	Infected cell protein 0
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate early
IFN	Interferon
IL	Interleukin
iPS	Induced pluripotent stem [cell]
IRF	Interferon regulatory factor
IR_L	Long internal repeat
IR_S	Short internal repeat
ISRE	Interferon-stimulated response element
JmjC	Jumonji domain C
JNK	c-Jun N-terminal kinases
K	Lysine
kb	Kilobase
kDa	Kilo Dalton
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	Latency associated nuclear antigen
LAT	Latency associated transcript
LMP	Latency membrane protein
LPS	Lipopolysaccharide
LSD1	Lysine-specific demethylase 1
LTP	Large tegument protein
m	Murine
MØ	Macrophage
MAP	Mitogen-activated protein
MAPP	Mitotic accumulation of PML protein
MART	Mono-ADP ribosyltransferase
mC-BP	mCP binding protein
mCP	Minor capsid protein
MCP	Major capsid protein

MCS	Multiple cloning site
MCM	Mini chromosome maintenance
MCMV	Murine CMV
MDBP	Methylated DNA-binding protein
me	Methylated
MEF	Murine embryonal fibroblast
MEKK	MAP Kinase Kinase Kinase
MgCl₂	Magnesium chloride
MHC	Major histocompatibility complex
MICB	MHC class I related chain B
MIEP	Major immediate early promoter
MIP	Macrophage inflammatory protein
miRNA	Micro RNA
MOI	Multiplicity of infection
MRF	Modulator recognition factor
mRNA	Messenger RNA
MS	Mass spectrometry
MSK	Mitogen- and stress-activated protein kinase
MSP	Methylation specific PCR
ND10	Nuclear domain 10
NIEP	Non-infectious enveloped particle
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NPC	Nasopharyngeal carcinoma
NT2D1	Human embryonal carcinoma cell line (T2)
Oct	Octamer transcription factor (see POU)
OD	Optical density
ORF	Open reading frame
oriLyt	Origin of lytic replication
p	Protein
PADI4	Peptidylarginine deiminase 4
PAGE	Poly-acrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
pAP	Precursor AP
PARP	Poly-ADP ribose polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase chain reaction
PDX1	Pancreatic and duodenal homeobox 1 / insulin promoter factor 1
PFA	Phosphonoformic acid (Foscarnet)
PFU	Plaque forming unit
PHD	Plant homeodomain
PKR	Protein kinase R
PMA	Phorbol 12-myristate 13-acetate (TPA)
PMNL	Polymorphonuclear leukocyte
PML	Promyelocytic leukaemia protein
PML-NB	PML nuclear body
pol	Polymerase
POU	Pit/Oct/Unc
POU2F1	POU class 2 transcription factor 1 (Oct1)
POU2F2	POU class 2 transcription factor 2 (Oct2)
POU3F1	POU class 3 transcription factor 1 (Oct6)
POU5F1	POU class 5 transcription factor 1 (Oct4 or Oct3/4)
pp	Phosphoprotein
pRb	Retinoblastoma protein
PRMT	Protein arginine methyltransferase
PRV	Pseudorabies virus
RA	Retinoic acid
RANTES	Regulated on activation, normal T cell expressed and secreted
RBCC	See TRIM
RBP-Jκ	J κ -recombination-binding protein
RC	Replication complex
RFP	Red fluorescent protein
RING	Really interesting new gene
RNA	Ribonucleic acid
RSK	Ribosomal S6 kinase
RT	Reverse transcription
RTQ	Real-time quantitative
SAM	S-adenosyl-L-methione
SBP	Silencing binding protein
SCMV	Simian CMV
SCP	Small capsid protein
Scr	Scramble
siRNA	Small interfering RNA
SMC	Smooth muscle cell
Sp100	Speckled protein of 100kDa

SSEA3	Stage-specific embryonic antigen-3
SUMO	Small ubiquitin-like modifier
SV40	Simian virus 40
T	Thymidine
T2	See NT2D1
T2RA	Retinoic acid induced differentiated form of NT2D1
TBP	TATA binding protein
TCID₅₀	Tissue culture infective dose 50%
TE	Tris-EDTA
TGF	Transforming growth factor
THP-1	Human acute monocytic leukemia cell line
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TPA	12- <i>O</i> -Tetradecanoylphorbol 13-acetate (PMA)
TRIM	Tripartite motif
TR_L	Long terminal repeat
TR_S	Short terminal repeat
TSA	Trichostatin-A
U	Uracil
U373 MG	Human glioblastoma astrocytoma cell line
UL/U_L	Unique long
US/U_S	Unique short
UV	Ultraviolet
vMIA	viral Mitochondrial inhibitor of apoptosis
VPA	Valproic acid
VSV	Vesicular stomatitis virus
VZV	Varicella-zoster virus
YY1	Yin Yang 1

1. Introduction

1.1 The *Herpesviridae*

The *Herpesviridae* are a numerous family of widely distributed large DNA viruses that naturally infect humans, other mammals and invertebrates (Minson *et al.*, 2000). Characteristics of herpesviruses include the self-provision of enzymes involved in nucleic acid metabolism, DNA synthesis and protein production; the synthesis of viral DNA and capsid assembly in the nucleus, whilst the final virion is produced in the cytoplasm; and production of infectious progeny virus, usually concomitant with destruction of the infected cell (Pellett & Roizman, 2007). However, the hallmark of the *Herpesviridae* lifecycle is latency whereby, after primary infection, the viral genome is able to persist in the absence of virus production.

Membership of the family *Herpesviridae* has historically been based on the architecture of the virion. However, formal taxonomy of the herpesviruses has been addressed since 1971 by the International Committee on Taxonomy of Viruses (ICTV; Wildy, 1971). A provisional approach to formally name herpesviruses was begun thereafter (Roizman *et al.*, 1973) and was followed with classification of the family into three subfamilies: *Alpha*-, *Beta*- and *Gammaherpesvirinae*, initially on the basis of biological characteristics (Roizman *et al.*, 1981). Further division into genera was then carried out using more precise genomic analysis (Roizman *et al.*, 1992) as well as through extensive molecular phylogenetic analysis, which included studies of size, genome structure and sequence comparisons (McGeoch *et al.*, 2000). Herpesvirus subfamilies have been further segregated into several genera (Davison *et al.*, 2005; see Fig. 1.1).

Over two hundred herpesviruses have now been classified into these three subfamilies, with an additional genus (*Ictalurivirus*) not attached to any subfamily and a large number still not assigned to specific genera (Davison, 2007). As outlined above, the *Herpesviridae* infect a range of species with all but one of the viruses assigned to taxa infecting either mammals or birds. In addition, a large number of unassigned herpesviruses infect lower vertebrate (fish, reptiles and amphibians) and invertebrate (bivalve) hosts (Davison, 2007). However, due to the extensive nucleotide sequence data from an increasing number of these divergent genera, a revision has been recently proposed by the Herpesvirus Study

Group of the ICTV: the entire taxon of herpesviruses is redefined under a new order, *Herpesvirales* (see Fig. 1.1). The *Herpesvirales* will then be split into three virus families: the *Alloherpesviridae* (fish and amphibians); the *Malacoherpesviridae* (bivalves); and the *Herpesviridae*, encompassing the herpesviruses of mammals, birds and reptiles as already recognised (reviewed in: McGeoch *et al.*, 2006).

1.2 The *Herpesviridae* subfamilies

As explained above, the members of the *Herpesviridae* were initially classified on the basis of their biological characteristics and they were further subdivided into subfamilies: the *Alpha*-, *Beta*- and *Gammaherpesvirinae*.

1.2.1 The *Alphaherpesvirinae*

The *Alphaherpesvirinae* share distinguishing qualities such as variable host range, short reproductive cycle, rapid spread in *in vitro* culture, efficient destruction of the infected cells and the ability to establish latency primarily within sensory ganglia. Main examples include Herpes Simplex virus-1 and -2 (HSV-1 & -2) from the *Simplexviruses*, which are the major causes of oral and genital herpes respectively and Varicella-Zoster virus (VZV), the cause of chicken-pox and shingles, from the *Varicelloviruses*. Both have mammalian hosts, whilst avian and reptilian hosts exist for the other members of the subfamily (see Fig. 1.1).

1.2.2 The *Betaherpesvirinae*

The *Betaherpesvirinae* subfamily is distinguished from others in the family by a long reproductive cycle and a slow infection rate in cell culture. Furthermore, the betaherpesviruses have a restricted host range, although this is not specific to the subfamily. A common biological characteristic is an enlargement of the infected cell (cytomegalia) and this feature is exemplified by the prototypic genus *Cytomegalovirus*. Other genera within the subfamily are *Muromegalovirus*, which includes murine cytomegalovirus (MCMV), and *Roseolovirus*, represented in the human host by human herpesvirus-6 (HHV-6) and which has been expanded to include the betaherpesviruses of mandrils and chimpanzees. The proposed *Proboscivirus* genus also includes those of large mammals such as endotheliotropic elephant herpesvirus-1 (EIHV-1). Across the range of

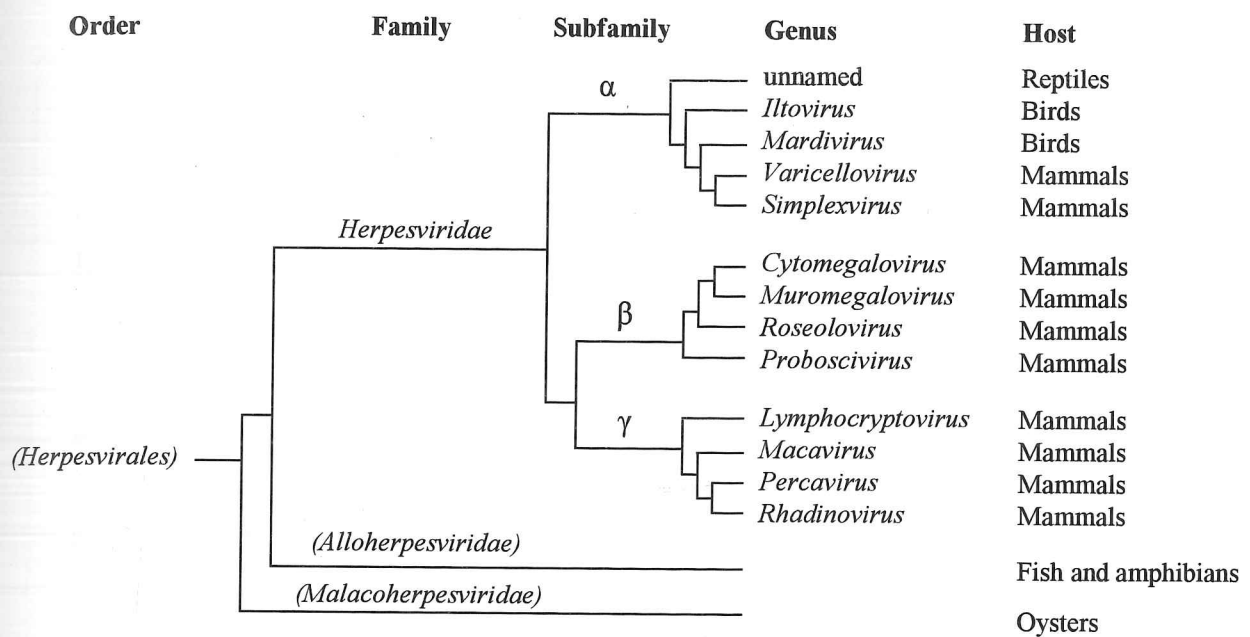


Figure 1.1 The *Herpesviridae* – major herpesvirus phylogenetic relationships and taxonomic subunits. The schematic shows branching patterns of all the assigned subfamilies and genera within the *herpesviridae*, including an unnamed genus with reptilian host species. In addition to the above scheme there is a further genus (*Ictalurivirus*) that remains unattached to any subfamily and a number of species that are not assigned to any genera (Davison & Bhella, 2007). Furthermore, labels shown in brackets are those from the proposed incorporation of the entire taxon into the order *Herpesvirales*. (Adapted from Fields Virology Fifth Ed.).

viruses within this subfamily, the virus can be maintained in latent form in kidneys, secretory glands and lymphoreticular glands amongst other tissues.

1.2.3 The *Gammaherpesvirinae*

Viruses of the *Gammaherpesvirinae* are usually specific for either T or B lymphocytes, although *in vitro* all members are able to replicate in lymphoblastoid cells (immature leukocytes) with some causing lytic infections in some types of epitheloid and fibroblastic cells. Latent virus is also often found in lymphoid tissue. The gammaherpesviruses are composed of two genera: the *Lymphocryptovirus*, includes the prototypic gammaherpesvirus Epstein-Barr virus (EBV) and the *Rhadinovirus* genus, exemplified by human herpesvirus-8 (HHV-8). Further, it has been proposed that the *Rhadinovirus* genus be expanded to include two new genera: the *Macavirus* genus infecting ruminants and the *Percavirus* genus hosted by perissodactyl (hoofed) and carnivore species (McGeoch *et al.*, 2006).

1.3 The Human Herpesviruses (HHVs)

Since herpesviruses are so highly disseminated in nature, it is not surprising that amongst mammalian species infected is the human. To date, there are eight main examples of herpesviruses known to infect the human host (with HHV-6 having two variants, A and B), representing all the major lineages populated by mammalian viruses (McGeoch & Davison, 1999). Indeed, the major human herpesviruses constitute the prototypic examples of each of the subfamily groups (see Table 1.1) and show a range of pathogenicity from oral and genital lesions (HSV-1 & -2), chickenpox and shingles (VZV) to cancer, such as Burkitt's lymphoma/nasopharyngeal carcinoma (EBV) and Kaposi's sarcoma (KSHV/HHV-8). However, some HHVs only show subclinical symptoms upon infection in the immunocompetent host, including HHV-6, -7 and the largest known human herpesvirus human cytomegalovirus (HCMV/HHV-5).

1.4 Human Cytomegalovirus (HCMV)

1.4.1 Introduction

Human Cytomegalovirus (HCMV), as the prototypic member of the *Betaherpesvirinae*, shares a number of the characteristics of this subfamily. These include salivary gland tropism, strict species specificity (which has hindered the establishment of animal models

<i>Designation</i>	<i>Common name</i>	<i>Abbreviation</i>	<i>Subfamily</i>	<i>Genome size (kbp)*</i>	<i>Sites of latency</i>	<i>Major associated diseases</i>
HHV-1	Herpes simplex virus type 1	HSV-1	α^S	152	Neurones (sensory ganglia)	Oral and genital herpes
HHV-2	Herpes simplex virus type 2	HSV-2	α^S	155	Neurones (sensory ganglia)	Genital and oral herpes
HHV-3	Varicella-zoster virus	VZV	α^V	125	Neurones (sensory ganglia)	Varicella (chickenpox) and zoster (shingles)
HHV-4	Epstein-Barr virus	EBV	γ^L	172	B lymphocytes	Burkitt's lymphoma, Hodgkin disease and nasopharyngeal carcinoma
HHV-5	Human cytomegalovirus	HCMV	β^C	230/236	Blood monocytes	Subclinical symptoms and congenital illness
HHV-6A	Human herpesvirus 6A	HHV-6A	β^{Ro}	159/170	Monocytes, T lymphocytes	Exanthem subitum (roseola) and febrile illnesses
HHV-6B	Human herpesvirus 6B	HHV-6B	β^{Ro}	162/168	Monocytes, T lymphocytes	Similar clinical symptoms to HHV-6A
HHV-7	Human herpesvirus 7	HHV-7	β^{Ro}	145	Unknown	Subclinical symptoms
HHV-8	Kaposi's sarcoma-associated herpes virus	KSHV	γ^{Rh}	170/210	B lymphocytes	Kaposi's sarcoma, Castleman's Disease, oral and genital lesions

^S Simplexvirus

^V Varicellovirus

^L Lymphocryptovirus

^C Cytomegalovirus

^{Ro} Roseolovirus

^{Rh} Rhadinovirus

* Genome size range reflects different strains.

Table 1.1 The Human Herpesviruses - classification and associated medical conditions.

for research purposes) and slow growth in cultured cells *in vitro* (Ho, 1991a; Ho, 2007; Mocarski *et al.*, 2007). The etiological appearance of HCMV infected cells was first formally described in the 1950s with cells typically demonstrating an enlarged cytomegalic appearance and the presence of intracellular inclusion bodies (Plummer, 1973; Roizman *et al.*, 1981; Rowe *et al.*, 1956; Smith, 1956; Weller *et al.*, 1957), although these had been described as early as 1881 without the knowledge of the associated agent (Ribbert, 1904). Indeed, HCMV was first known as salivary gland virus due to its tropism until the *Cytomegalovirus* nomenclature was proposed and the virus finally named human cytomegalovirus (Weller *et al.*, 1957).

1.4.2 Clinical features of HCMV

HCMV is a ubiquitous virus infection with seroepidemiological studies demonstrating worldwide distribution of the virus amongst human populations. The prevalence of infection is largely due to the success of the virus as a persistent pathogen with efficient mechanisms of transmission but is also affected by socio-economical factors: developed countries have levels from 40-60% seroprevalence whereas areas of the developing world have seroprevalence levels as high as 100%.

Primary infection with HCMV is often asymptomatic but results in lifelong persistence in the host. Infected individuals are therefore able to pursue a normal life but persistence provides the virus with a higher chance of transmission through bodily secretions at times of viral shedding (Ho, 1990). It has been shown that infants are able to shed large quantities of virus in both saliva and urine for long periods of time following infection, providing an opportunity for transmission of the virus, with hand washing following contact with infants reducing the risk of HCMV transmission considerably (Adler, 1986a; Adler, 1986b). In addition, the transmission of HCMV can occur vertically as well as horizontally. Mothers are able to pass on HCMV to their foetus *in utero* and during passage through the birth canal, resulting in a number of congenital defects in newborns, and also to their children through breast feeding or via saliva or urine contact (Numazaki *et al.*, 1970; Pass, 1985; Stagno *et al.*, 1986). In addition to infection during the perinatal period (0-2 years), horizontal transmission after puberty through sexual contact represents the second most important natural mode of transmission and both comprise the key times

associated with the natural acquisition of HCMV (Drew *et al.*, 1981; Chandler *et al.*, 1985; Handsfield *et al.*, 1985; Doerr, 1987).

A major feature of the HCMV lifecycle, particular to all herpesviruses, that aids transmission of the virus is persistence of the virus in the infected individual due to the period of latency, whereby the viral genome is maintained in the absence of virus production (Sinclair & Sissons, 2006). The virus can then be transmitted through transplantation of solid organ or bone marrow tissues and following whole blood transfusion from a seropositive individual and can result in severe life-threatening disease (Rubin, 1990; Miller *et al.*, 1991).

1.4.3 HCMV associated disease, treatment and prevention

The number of syndromes associated with HCMV infection, whether reactivation or primary infection, in addition to the resultant cost of treating patients undergoing prophylactic or pre-emptive therapy has led to the National Institutes of Health (NIH) designating HCMV as one of the major targets for extended research.

Acute pathogenicity of HCMV infection is usually only observed in the absence of an effective host immune response. Complications can arise in individuals whose immune system is immature: congenital infection of the immature foetus can cause a major risk to the development and long term health of the baby causing such conditions as hearing loss, jaundice and mental retardation through encephalitis in the womb (Boppana *et al.*, 1992; Raynor, 1993). However, HCMV also represents a significant opportunistic pathogen of immunocompromised individuals. Indeed, HCMV was first recognised as an opportunistic pathogen in immunocompromised patients soon after the introduction of allograft transplantation (Rifkind, 1965; Singh *et al.*, 1988; Ho, 1991b; Rubin, 2001). Problems were observed with individuals who were immunocompromised by suppression with drug treatment, including virus reactivation in transplant recipients but also transfer from latently infected allograft donors (Adler, 1983; Drew, 1988; Rubin, 1990; Reinke *et al.*, 1999). HCMV infection in renal transplant recipients is a known major clinical problem, with both short and long term sequelae and the importance of recipient and donor serostatus well recognised: seronegative patients are at greatest risk after transplantation

from a seropositive donor, although reactivation in a seropositive recipient can also cause a major health risk (Hughes *et al.*, 2008).

Primary infection and reactivation of latent virus has also begun to pose a serious health threat, more recently, in HIV carriers who eventually develop acquired immunodeficiency syndrome (AIDS); up to 90% of patients now develop an active or invasive HCMV infection (Jacobson & Mills, 1988) with HCMV infection symptoms becoming one of the hallmarks of late stage AIDS. However, severe infection is now becoming less common with active retroviral therapy (ART), including anti-virals directed at herpesviruses, being used prophylactically. Indeed, due to the increase in resistance to pharmaceuticals against herpesvirus infections, several treatments are now available targeting different constituents of the viruses and their lifecycle. The main treatments for HCMV are ganciclovir (GCV), cidofovir (CDV) and foscarnet (PFA) which target the viral DNA polymerase (UL54) and are used for severe life threatening disease (Landolfo *et al.*, 2003). New treatments have focussed on disabling the activity of other components of the virus, such as proteases, including the drug maribavir which was fast-tracked by the US Food and Drug Administration (FDA) into clinical trials and production due to its activity against the UL97 gene product (Biron *et al.*, 2002; Drew *et al.*, 2006). However, it is becoming apparent that resistance is now occurring towards this new era of anti-HCMV drugs such that novel targets and pharmaceuticals will become necessary with time.

Rather than treatment of HCMV disease, it has been hypothesised that prevention of disease in the first instance should be possible. High risk groups are also known, which could aid targeting of sub-populations for vaccination to reduce the risks of disease in later life. There are no apparent obstacles to immunisation against HCMV with data supporting the feasibility of this form of prevention (Adler, 2008). The guinea pig model of congenital CMV has provided good evidence that both active and passive immunisation are effective at reducing pup mortality and congenital infections using live attenuated virus, killed virus and recombinant vaccines or immune sera (Bia *et al.*, 1984; Schleiss *et al.*, 2002; Schleiss *et al.*, 2007). Indeed, it is also known that immunity is induced during natural infection of the human host, which reduces the rate of re-infection in immunocompetent adults (Plotkin *et al.*, 1989; Adler *et al.*, 1995), and that women can be passively immunised when infected during pregnancy (Nigro *et al.*, 2005). Hence, the likelihood for positive results

from human immunisation is high. This has led to the production of two vaccines currently in phase II clinical trials. The gB/MF59 vaccine is based on the major glycoprotein of HCMV administered with the adjuvant MF59 which has been shown to create high levels of neutralising antibodies in animals and humans and stimulate the cytotoxic T lymphocyte response (Mitchell *et al.*, 2002). Secondly, the second vaccine is based on the live attenuated HCMV Towne strain which is not able to reactivate in either healthy or immunosuppressed patients. It is able to stimulate neutralising antibodies and cellular immune responses, and has also shown protection for renal transplant patients from severe post-transplantation HCMV disease (Adler *et al.*, 1998; Jacobson *et al.*, 2006). However, due to difficulties in carrying out trials and the lack of public awareness of HCMV infection and disease, the production of a vaccination applicable to the human population is yet to be formally produced.

1.5 HCMV structure and life cycle

1.5.1 HCMV structure

The mature HCMV virion ranges in size from between 150nm to 250nm in diameter and, as with other herpesviruses, contains a single linear, double stranded DNA (dsDNA) genome (Geelen *et al.*, 1978a; Geelen *et al.*, 1978b; Irmieri & Gibson, 1985; Butcher *et al.*, 1998). The genome is encapsidated within an icosahedral nucleocapsid, 100nm in size (McGavran & Smith, 1965; Irmieri & Gibson, 1985; Butcher *et al.*, 1998), which is coated in a tegument layer composed of a number of different proteins underlying the viral envelope, which has numerous viral glycoproteins embedded within it (Spaete *et al.*, 1994; Britt & Mach, 1996) (Fig. 1.2). This layered structure is composed of around 35 different proteins with over 40% of the total protein mass and a number of key viral proteins being found in the tegument including the phosphoproteins pp65 (UL83), pp71 (UL82) and pp28 (UL99) (Irmieri & Gibson, 1983; Roby & Gibson, 1986). Although the functions of these phosphoproteins are still being fully elucidated, a number of them appear to be involved in transactivation of viral gene expression (Liu & Stinski, 1992; Winkler & Stamminger, 1996; Homer *et al.*, 1999; Schierling *et al.*, 2004; Cantrell & Bresnahan, 2005; Cantrell & Bresnahan, 2006; Saffert & Kalejta, 2006; Hwang & Kalejta, 2007) or in the disruption of host cell processes prior to *de novo* protein synthesis (Hoffman *et al.*, 2002; Kalejta *et al.*, 2003; Kalejta & Shenk, 2003a; Kalejta & Shenk, 2003b). A number of the viral and, indeed, cellular proteins that compose an HCMV virion have been identified by

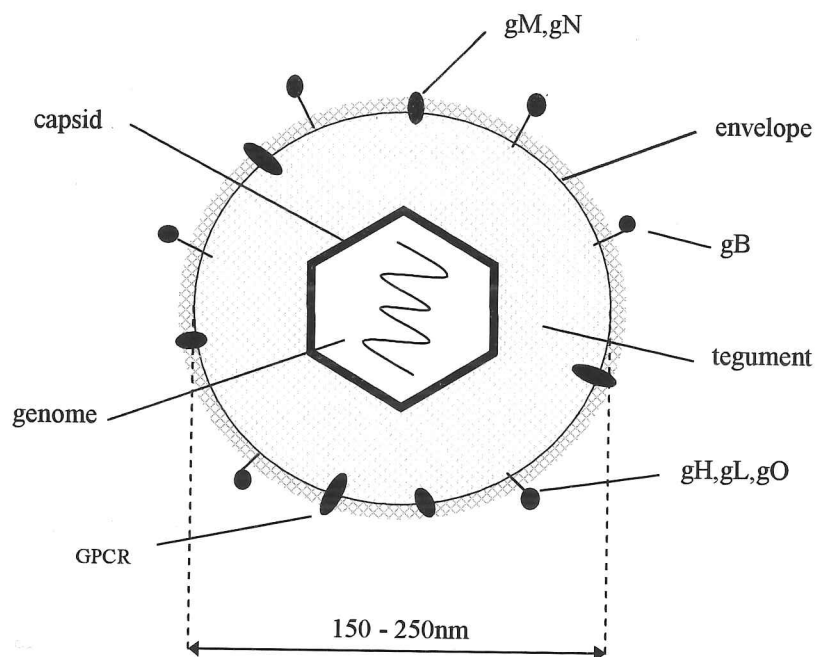


Figure 1.2 The HCMV virion structure. The HCMV infectious virion ranges in size from 150-250nm in diameter. It is comprised of a 230-236kbp linear double stranded DNA (dsDNA) genome encapsulated in an icosohedral capsid structure. The capsid is surrounded by a layer of tegument which is comprised of a number of phosphoproteins including pp71 and pp65. Enclosing the tegument is a lipid bilayer covered in a number of glycoproteins and glycoprotein complexes (gX) necessary for mediating virus entry into the target cell and the G-protein coupled receptor (GPCR).

biochemical and immunological approaches (Baldick & Shenk, 1996; Gibson, 1996). The cellular component of the virion includes CD13 aminopeptidase N, β_2 -microglobulin, protein phosphatase I, annexin II and actin-related protein 2/3 (Arp2/3) (Baldick & Shenk, 1996; Giugni *et al.*, 1996; Grundy *et al.*, 1987; Michelson *et al.*, 1996; Stannard, 1989; Wright *et al.*, 1995). However, more recently developed sophisticated liquid phase separations and mass spectrometry (MS)-based approaches for analysing complex mixtures of polypeptides has allowed more detailed and total analysis of the proteome of the mature virion (Varnum *et al.*, 2004).

1.5.2 HCMV genome organisation

The HCMV genome has a predicted size of 230kbp based on analysis of the laboratory strain AD169 (Chee *et al.*, 1990a) and is maintained in a linear formation and enclosed within the viral nucleocapsid of the infectious virion (McGavran & Smith, 1965). The sequence analysis initially performed on AD169 identified 208 possible open reading frames (ORFs), which encoded for over 100 amino acids, although only 189 were thought to be unique due to duplication within the R_L region of the genome. The ORFs were numbered sequentially with the genome being organised into unique short and long regions (U_S and U_L) flanked by inverted short and long terminal repeats (TR_S and TR_L) and further internal repeats (IR_S and IR_L) at the L-S junction (Fig. 1.3). However, additional analysis of the Toledo strain has found differences to the high passage AD169 strain that had been extensively passaged in the laboratory to create a live attenuated vaccine (Elek & Stern, 1974). A 15kbp region named the U_Lb' region, not present in the AD169 strain, and a further 929bp (present in AD169) affecting UL42 and UL43 exist within the genome (Cha *et al.*, 1996; Dargan *et al.*, 1997). This finding has also been confirmed with analysis of the wild-type clinical isolate Merlin (Dolan *et al.*, 2004). Further comparison and alignment analysis of AD169 to chimpanzee CMV (CCMV) has also led to a re-evaluation of the gene complement, which is more likely around 145 ORFs, with a wild type HCMV complement probably between 164-167 ORFs (Davison *et al.*, 2003). However, additional alignment analyses and coding potential re-evaluation of low passage strains, such as Toledo, against other recent clinical isolates (FIX/VR1814, PH and TR) has demonstrated up to 252 possible ORFs and multiple sequence alignments revealing substantial variation in the amino acid sequences encoded by many of the conserved ORFs (Murphy *et al.*, 2003a; Murphy *et al.*, 2003b). In addition, as well as providing further sequence data for

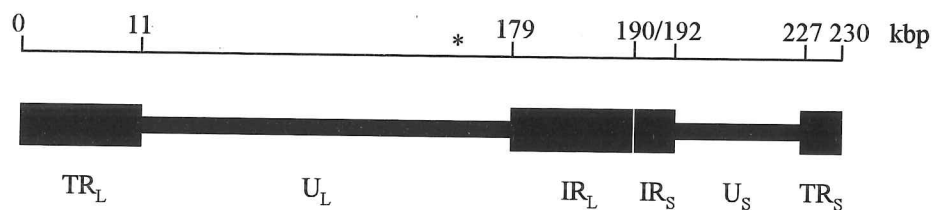


Figure 1.3 HCMV genome organisation. HCMV has a 230-236kb double stranded DNA genome. It comprises both long (_L) and short (_S) terminal repeats (TR), internal repeats (IR) and unique regions (U) where the majority of the coding ORFs reside. The HCMV genome pictured above is that of the highly passaged laboratory strain AD169. However, the genome of other clinical isolates, such as HCMV Toledo and TB40/E, have much shorter TR_L and IR_L regions. The latter region is replaced by a unique region (U_Lb') known to encode multiple immune evasion genes found unnecessary for AD169 replication in fibroblasts. (* = position of the HCMV MIEP).

the HCMV strain TB40/E, recent work has also shown that the plaque purified form of this virus may actually contain three separate clones (Sinzger, *et al.*, 2008).

1.5.3 HCMV entry

Although certain human cell types do not fully support HCMV replication, entry of HCMV into cells appears to be promiscuous with the virus capable of binding and penetrating virtually all vertebrate cell lines used commonly (Nowlin *et al.*, 1991). Entry of HCMV into cells such as fibroblasts takes place via two defined stages, both involving sequential interactions between viral and cellular components and being fully pH-independent. First, the virus binds to cell surface in a tethering step to heparin sulphate proteoglycans via glycoprotein B (gB) and/or the gM/gN heterodimer on the viral envelope (Kari & Gertz, 1992; Compton *et al.*, 1993; Compton, 2004). This interaction is then secured through docking of gB with a further receptor or receptors, which due to the current widely differing opinions in the field will be discussed further below. Through gB, and via interactions with other glycoproteins (gH/gL/gO) and probably cellular integrins, fusion of the viral envelope with the cellular membrane occurs and thus entry of the virion (Compton *et al.*, 1992; Compton, 2004; Kinzler & Compton, 2005).

Although surface fusion of the virion in cells such as fibroblasts remains the principal mechanism by which HCMV is thought to infect cells, it has now become apparent that HCMV is also able to enter biologically relevant epithelial and endothelial cell types in a pH-dependent fashion following an endocytic route. Work from a number of groups using the highly passaged AD169 strain and low passage clinical isolates has shown that proteins encoded by viral genes UL128, UL130 and UL131 form a complex with the gH/gL heterodimer and are necessary for adsorption to, and penetration of, epithelial cells and also promote the early stages of virus replication (Wang & Shenk, 2005a; Wang & Shenk, 2005b; Ryckman *et al.*, 2006; Jarvis & Nelson, 2007; Ryckman *et al.*, 2008; Sinzger *et al.*, 2008; Sinzger, 2008). It has also been proposed that the cell type that the HCMV particle is produced in also influences its subsequent spread, due most likely to a change in the balance of glycoprotein complexes found on the surface of the envelope (Wang *et al.*, 2007) and that antibodies from human serum directed specifically at UL128/UL130/UL131 can inhibit infection of epithelial and endothelial infection whilst infection of fibroblasts is unaffected (Gerna *et al.*, 2008). Ultimately, however, the viral capsid is still released from

the virion through fusion of the envelope, although in this case through a pH-dependent mechanism with the internal lipid bilayer of the host endosome.

Upon uncoating of the virion, the capsid is released into the cytosol along with numerous associated tegument proteins (Fig. 1.4). The viral genome is delivered to the nucleus by the capsid after trafficking to a nuclear pore, possibly involving viral proteins and likely facilitated via cytoplasmic microtubules (Dohner & Sodeik, 2005), where it is released at a nuclear pore, most likely aided by the large tegument protein (LTP, UL48) and a binding protein (LTPbp, UL47) (Dunn *et al.*, 2003; Yu *et al.*, 2003). The genome then rapidly circularises and may be deposited at or near nuclear domain-10 (ND10) bodies, with increasing evidence from the herpesvirus family supporting this (Maul, 1996; Maul, 1998; Everett *et al.*, 2007). It is at this point that the permissiveness for infection is then determined: it is known that in non-permissive cell types the prevention of productive infection is exerted after viral entry and that in model cell systems the viral genome can be found in a nuclear location within these cells (Nelson & Groudine, 1986).

As alluded to above, much work has focussed on finding the receptor(s) necessary for HCMV entry. During HCMV infection, considerable changes in the metabolic state of the cell occur. These changes also occur upon infection with UV-inactivated virus (Fortunato *et al.*, 2000; Compton, 2004). Consequently, the process of binding of the virus to the cell surface is sufficient to result in significant homeostatic and transcriptional changes within the cell prior to *de novo* viral protein synthesis (Boldogh *et al.*, 1991b; Kowalik *et al.*, 1993; Yurochko *et al.*, 1995; Boyle *et al.*, 1999). Signalling pathways that appear to be affected have prompted the investigation of the epidermal growth factor (EGF) response, as internalisation of the EGF-receptor (EGF-R) results from virus binding. Consistent with this, it was shown that an EGF-R null breast cancer cell line was resistant to HCMV infection but that the reintroduction of EGF-R through transfection led to susceptibility to infection (Wang *et al.*, 2003). Furthermore, this investigation also found that gB co-immunoprecipitated with EGF-R, suggesting an active role for EGF-R in HCMV entry. However, other studies have determined a different mechanism for the interaction between HCMV and EGF-R. It was observed that HCMV inhibited signalling via EGF-R and also that infection caused a down-regulation of transcription and resultant cell surface expression of EGF-R, although this was dependent on viral gene expression and therefore

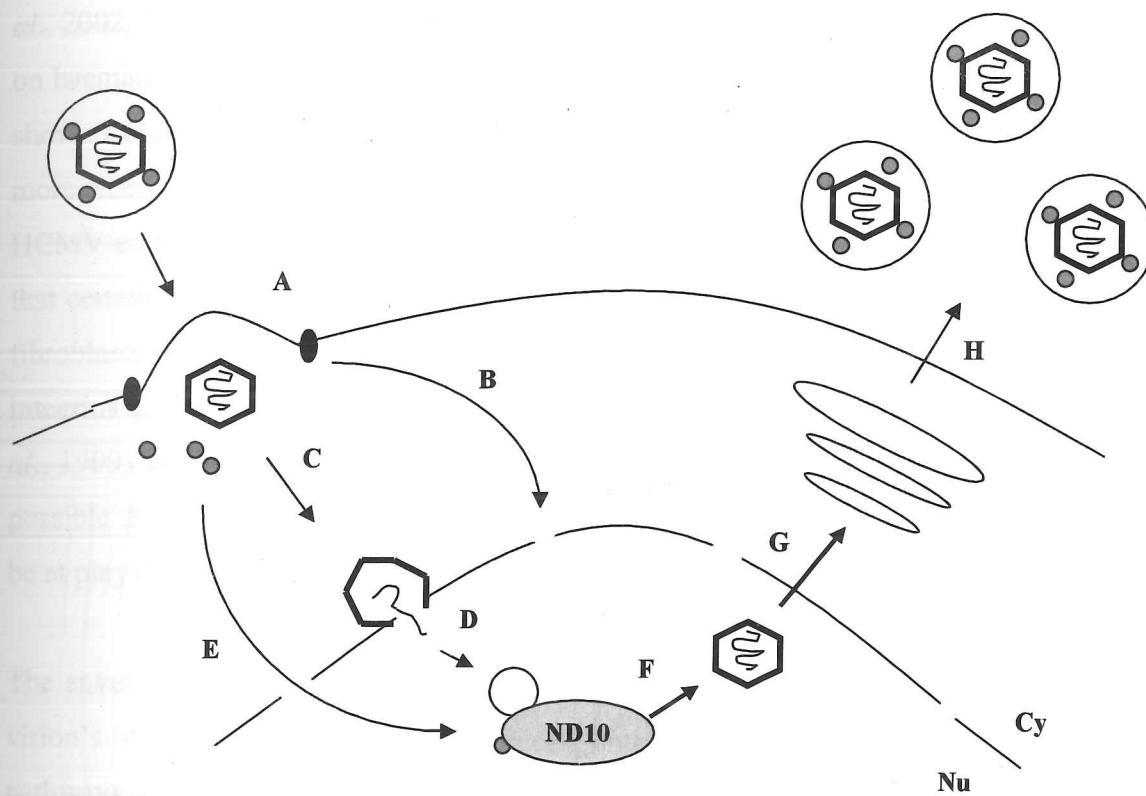


Figure 1.4 The HCMV life cycle. (A) HCMV enters cells via attachment to and fusion of the cellular lipid membrane. (B) Initial attachment leads to intracellular signalling, (C) whilst the capsid transits to the nucleus. (D) The genome is released at a nuclear pore and circularises before association of ND10 components with the episome or deposition of the episome at ND10 bodies occurs. (E) Tegument proteins traffic to ND10 domains where they can act as transactivators of viral gene expression. (F) In productive infection, a temporally regulated cascade of gene expression occurs alongside genome replication, leading to packaging in the nucleus. (G) Capsids exit the nucleus via an envelopment-deenvelopment pathway, (H) before acquiring a final envelope at a late cytoplasmic compartment and egress from the cell. (Cy = cytoplasm; Nu = nucleus).

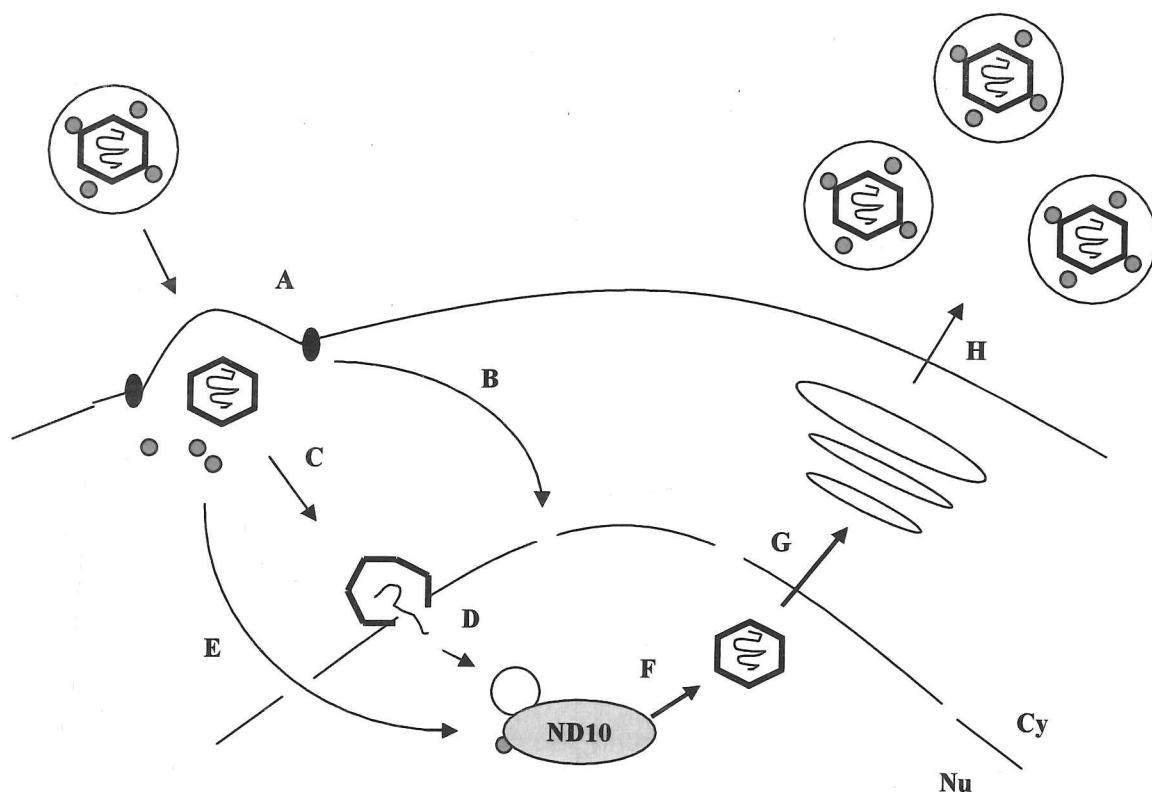


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argued for a different mechanism of EGF-R regulation at later times of infection (Fairley *et al.*, 2002; Beutler *et al.*, 2003). It is important to note, though, that EGF-R is not expressed on haematopoietic monocytes, macrophage or dendritic cells (DC), all of which have been shown as sites of primary infection or latency with HCMV (Ewald *et al.*, 2003). Indeed, more recent work has shown that EGF-R does not appear to have any role in the process of HCMV entry into fibroblast, epithelial or endothelial cell lines (Isaacson *et al.*, 2007) but that certain $\beta 1$ and $\beta 3$ integrin heterodimers are critical mediators of entry into permissive fibroblasts through interaction with gB (Feire *et al.*, 2004). However, interactions between integrins and EGF-R are known to occur on the cell surface (Miyamoto *et al.*, 1996; Li *et al.*, 1999) and since integrins appear to be indispensable for infection of fibroblasts, it is possible that multiple mechanisms, including the need for EGF-R in some cell types, may be at play during cell entry of HCMV.

The envelope proteins of HCMV, as explained above, are an important component of the virion's structure with involvement in cell entry, cell activation, changes in signalling pathways and interaction with the immune system. The glycoprotein gB had been thought to be predominant on the surface of the virion, most likely due to its multifunctional properties in cell entry, cell to cell transmission through fusion and signal pathway transduction (Britt & Mach, 1996; Compton *et al.*, 1993; Navarro *et al.*, 1993). In addition, gB is also known to bind cellular annexin II indicating a possible role in trafficking and egress of the virus (Pietropaolo & Compton, 1997; Pietropaolo & Compton, 1999; Singh & Compton, 2000). However, it is important to note that recent evidence suggests that the levels of gB on a virion surface are much lower than first thought, being just 1% of the total virion protein content, whereas gM (UL100) displays a much higher proportion (Varnum *et al.*, 2004). However, gB remains a major target for neutralising antibodies in the serum of infected patients (Navarro *et al.*, 1997) and this has led to the identification of several gB epitopes that have been important for collection of epidemiological data on the spread of HCMV worldwide (Chou & Dennison, 1991; Chou, 1992; Fries *et al.*, 1994). Indeed, gB has been one of the primary candidates for the development of an HCMV vaccine (Spaete, 1991; Marshall *et al.*, 1992; Gonczol *et al.*, 1995; Pass *et al.*, 1999). Neutralising antibodies directed at the major glycoprotein complex of gH/gL/gO also have profound effects on viral penetration and transmission indicating that these proteins are also essential for viral entry (Rasmussen *et al.*, 1984; Keay & Baldwin, 1991).

1.5.4 HCMV DNA replication

HCMV is able to productively infect a number of different cell types including fibroblast, epithelial, endothelial, smooth muscle, macrophage and dendritic cells, dependent upon viral strain (Lathey *et al.*, 1990; Ibanez *et al.*, 1991; Sinzger *et al.*, 1993; Sinzger *et al.*, 1995; Riegler *et al.*, 2000). After deposition of the genome in the nucleus and circularisation, which is thought to occur within the first four hours, viral gene expression is initiated (discussed fully in section 1.6). Although many viruses rely on the host transcriptional machinery to replicate their own DNA, HCMV encodes a number of proteins to carry out this process (Huang, 1975; Anders *et al.*, 1986; Anders & McCue, 1996) and the replication of viral DNA is ultimately dependent on the expression of these proteins (White & Spector, 2006). Replication of viral DNA begins via directional rolling circle replication at around 18 hours post-infection and peaks at 60-80 hours post-infection (Stinski, 1978) resulting in the production of several thousand copies of viral DNA in replication compartments. Replicating viral DNA is generally greater in size than genome unit length and lacks terminal fragments, consistent with rolling circle replication (LaFemina & Hayward, 1983; McVoy & Adler, 1994) and not a limited theta replication form hypothesised by some. Replicated viral genomes are eventually packaged and released in mature virions over a further period of 48 hours (Stinski *et al.*, 1979; Penfold & Mocarski, 1997).

HCMV encodes a number of conserved proteins that bind the viral replication fork, including a DNA polymerase (UL54), an associated processivity factor (UL44), a single strand DNA binding protein (UL57) and a helicase constructed from three sub-units (UL70, UL102 and UL105) (Anders & McCue, 1996; Sarisky & Hayward, 1996). These proteins are able to bind to the viral lytic origin (*oriLyt*) and function to initiate replication with some additional viral gene products (see below) (Prichard *et al.*, 1996). The viral DNA polymerase is one of the most conserved proteins across the *herpesviridae* family and, as such, has become a significant target for anti-viral therapeutics, including ganciclovir (GCV) (Freitas *et al.*, 1985). HCMV does not encode a thymidine kinase (Estes & Huang, 1977), as other herpesviruses such as HSV-1 and -2 do and is, therefore, less susceptible to nucleotide analogues as anti-virals. However, it does express a kinase (UL97) which is able to use GCV as a substrate (Littler *et al.*, 1992) leading to the accumulation of the triphosphate form of GCV which when incorporated into replicated

DNA causes termination of DNA strand extension (Sullivan *et al.*, 1992). However, it is now known that many point mutations in the DNA polymerase of HCMV and in the UL97 gene product are able to render this drug ineffective (Chou *et al.*, 1997; Smith *et al.*, 1997). Therefore, other drugs have been generated to combat this problem, including cidofovir (CDV) (Xiong *et al.*, 1996) and foscarnet (PFA) (Wahren *et al.*, 1985) as well as more bio-available forms of GCV such as valGCV. In addition, other drugs such as maribavir, which specifically acts on the serine/threonine kinase UL97, shown recently to stabilise pRb during infection (Prichard *et al.*, 2008), are now in clinical trials (Drew *et al.*, 2006), although it is again becoming apparent that resistance can still form even to these new drugs (Chou *et al.*, 2007).

Other viral proteins also appear indispensable for viral DNA replication. Some immediate early (IE) proteins, such as TRS1/IRS1, UL84 and the UL112 and UL113 gene products are also important (Pari *et al.*, 1993; Pari & Anders, 1993; Iskenderian *et al.*, 1996). It is possible that the major IE and TRS1/IRS1 proteins act as transactivators for the transcription of the DNA replication machinery and therefore are necessary indirectly for producing a host cell environment conducive to DNA replication (Pari *et al.*, 1993). However, it has been suggested that the UL84 gene product is much more directly involved in the process due to its ability to sequester the six herpesvirus-conserved replication fork proteins and drive replication from the oriLyt sequence (Sarisky & Hayward, 1996): UL84 mutant viruses are defective for viral synthesis and growth (Xu *et al.*, 2004). The four UL112-UL113 encoded phosphoproteins (pp34, pp43, pp50 and pp84), produced as a result of alternate mRNA splicing (Wright *et al.*, 1988; White & Spector, 2006), are also critical during initiation of viral DNA synthesis (Park *et al.*, 2006). All four proteins form a complex, accumulate compartmentally prior to replication initiation (Ahn *et al.*, 1999) and are essential for the targeting of UL44 and the replication machinery to the viral genome at the pre-replication foci near ND10 (Penfold & Mocarski, 1997).

1.5.5 HCMV assembly, maturation and egress

The HCMV capsid is first assembled in the nucleus from the five viral proteins: the major capsid protein (MCP; UL86), the minor capsid protein (mCP, UL85), the assembly protein (AP) precursor (pAP, UL80.5), the smallest capsid protein (SCP, UL48.5) and the mCP

binding protein (mC-BP, UL46) (Gibson, 1996). The capsid is composed of 162 capsid polypeptides arranged as hexons and pentons in an icosahedral lattice structure studded with the SCP (Streblow *et al.*, 2006). A MCP:AP (1:1) precursor complex controls the formation of capsids that initially associates with an pAP conserved carboxyl terminal domain following synthesis at cytoplasmic sites and is responsible for translocating the MCP to the nucleus (Plafker & Gibson, 1998). This initiates the capsid assembly process. Within the nucleus, a conserved amino terminal pAP domain then facilitates oligomerisation which drives the formation of the MCP:AP complex described above before the SCP caps the structure and a number of protease cleavage steps separate AP from MCP. This inactivates the protease, completing the formation of the so called B capsid. Once assembled, it is thought that these immature particles are then transported to DNA replication compartments through direct interaction with viral proteins UL93 and UL52. These fibrillar networks can then be seen as dense inclusions by light microscopy (Fons *et al.*, 1986). Although much work has elucidated the various proteins involved in the encapsidation of the HCMV genome after replication, this process is best understood through observations with the alphaherpesvirus HSV-1. Upon HCMV DNA synthesis, encapsidation proteins recognise the concatemeric genomes through conserved *cis*-acting elements located within the terminal sequence of the viral genome. One full length genome is inserted into the capsid, a junction sequence is then recognised and cleavage of the genome occurs, producing a nucleocapsid containing a single viral genome. This modified 'head-full' packaging process is reminiscent of that with bacteriophage (Yamagishi & Okamoto, 1978). In total, ten herpesvirus core genes (UL51, UL52, UL56, UL77, UL89, UL93, UL95, UL97, UL103, and UL104) influence encapsidation and include elements of the packaging machinery and all are essential for HCMV replication (Mocarski *et al.*, 2007). The insertion of genome into so-called type B capsids displaces assembly protein (UL80.5) and causes the capsids to locate to a thickened concave area whereupon egress can begin to occur (Gibson, 1996).

Viral egress occurs around two days after replication has been completed (Penfold & Mocarski, 1997). Initially, nucleocapsids collect in the nucleus before budding into the perinuclear space (Fons *et al.*, 1986; Gibson, 1996). This is facilitated by recruited cellular kinases that dissolve the nuclear lamina (Muranyi *et al.*, 2002). Egress from the nucleus requires two core functions, the UL50 and UL53 gene products (Mettenleiter, 2004). It is

still unclear how the development of the HCMV coat and release of the mature virion takes place: indeed, the topic is contentious with exit from the nucleus and acquisition of the envelope both in question (reviewed in: Campadelli-Fiume *et al.*, 2006). However, it has been generally accepted that this does occur at least through a process of envelopment and de-envelopment similar to that seen with HSV-1, where the perinuclear envelope is lost during maturation with acquisition of the final envelope from a cytoplasmic compartment (Skepper *et al.*, 2001), and recent work with gO deletion viruses has shown the necessary presence of this glycoprotein during egress and secondary envelopment within multiple cell lines (Jiang *et al.*, 2008). The release of viral progeny then occurs in vesicles via the Golgi apparatus and secretion from the surface of the cell (Eggers *et al.*, 1992).

In addition to mature infectious virions, HCMV infected cells generate two further types of particle. Non-infectious enveloped particles (NIEPs) are composed of the same viral proteins as infectious virions but lack viral DNA, and are distinguishable from infectious particles by electron microscopy (EM) due to an electron dense core (Mocarski *et al.*, 2007). Secondly, a uniquely characteristic particle produced during HCMV and simian CMV (SCMV) infection is the dense body (DB), a non-replicating fusion competent particle composed predominantly of the tegument protein pp65 (UL83). The quantities and ratios of each of these different particles produced during an infection are dependent on the viral strain but also on the multiplicity of infection (Irmieri & Gibson, 1983; Gibson & Irmieri, 1984; Gibson, 1996). Importantly, clinical isolates of the virus accumulate fewer dense bodies and less pp65 than laboratory strains do (Klages *et al.*, 1989) and dense bodies are not observed in tissues or during infection of fibroblasts with a pp65 mutant virus (Schmolke *et al.*, 1995). Due to the absence of a packaged genome in DBs, and thus the inability to cause an infection *per se*, DBs have been touted as a vehicle for vaccination. As pp65 is one of the major antigens during infection (Reddehase, 2002) a new direction has been to produce DBs constructed from pp65 fused to further major antigens, such as IE72 (Mersseman, *et al.*, 2008).

1.6 HCMV productive infection

1.6.1 Immediate Early (IE) gene expression

In permissive cell types, viral transcription initiates with the expression of the IE genes, sometimes referred to as the alpha genes (α) (see Fig. 1.5) and has been mapped to four

regions: UL36 and UL37, UL122 and UL123 (IE72 and IE86), TRS1 and IRS1 and US3. All of these regions give rise to multiple transcripts and gene products. The US3 region gives rise to gene products associated with inhibition of trafficking of MHC class I molecules to the plasma membrane (Ahn *et al.*, 1996; Jones *et al.*, 1996; Mocarski, 2004). The UL36 and UL37 ORFs have multifunctional gene products: in addition to UL37x3 being associated with gene regulation activities (Hayajneh *et al.*, 2001), UL37x1 encodes a cell death suppressor denoted viral mitochondrial inhibitor of apoptosis (vMIA) (Goldmacher *et al.*, 1999; Goldmacher, 2002; Goldmacher, 2005). Furthermore, UL36 encodes a betaherpesvirus-conserved cell death suppressor, the viral inhibitor of caspase 8 activation (vICA) (Skaletskaya *et al.*, 2001; McCormick *et al.*, 2003; Goldmacher, 2005). The two gene products of the U_s regions IRS1 and TRS1, in addition to their ability to regulate gene expression (Blankenship & Shenk, 2002), are able to inhibit IFN-inducible protein kinase R (PKR) (Child *et al.*, 2004). Although UL36, UL37, UL37x1, TRS1 and IRS1 are all individually dispensable for viral replication, a number of mutants have been shown to be highly affected by a loss of two or more of these gene products, demonstrating the multifunctional and necessary nature of these proteins for virus infection (Mocarski *et al.*, 2007).

The expression of the major IE (MIE) gene products IE72 and IE86, sometimes termed IE1 and IE2 respectively, is driven by the major immediate early promoter/enhancer (MIEP), independent of *de novo* protein synthesis (Wathen *et al.*, 1981; Wathen & Stinski, 1982). The MIEP is one of the strongest known promoters and is responsible for the high levels of IE72 and IE86 RNA, generated from differential splicing of a common primary transcript, within 3 hours of infection. IE protein expression from this locus is critical for the regulation of the temporal cascade of viral gene expression typically associated with herpesviruses and is essential for the initiation of the early (E) phase of viral gene expression (Spector, 1996). IE72 and IE86 have a central regulatory role in HCMV infection (Colberg-Poley, 1996; Stenberg, 1996; Fortunato *et al.*, 2000; Castillo & Kowalik, 2002; Goldmacher, 2004) and, as such, the multifunctional importance of these two proteins will be discussed later (see section 1.9.2).

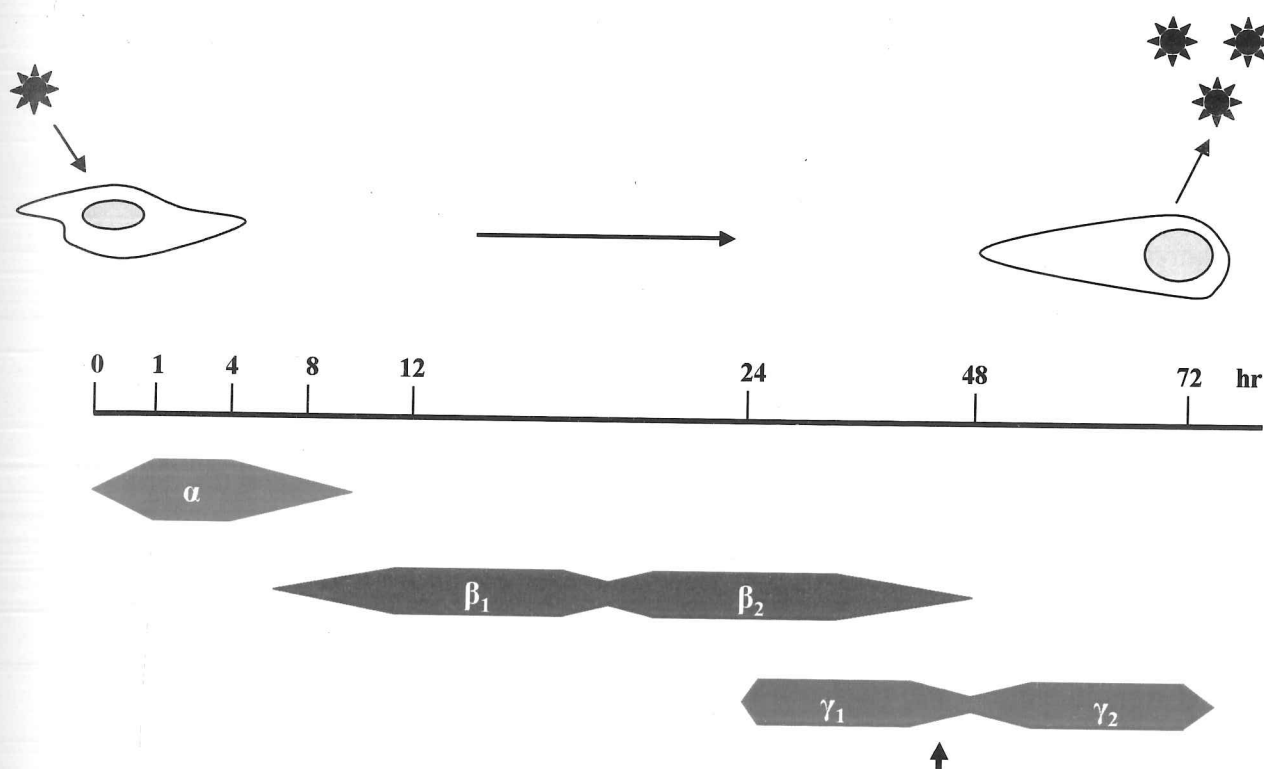


Figure 1.5 The temporal cascade of HCMV gene expression. Following infection of a permissive cell with HCMV, alpha (α) gene expression can be detected as early as 1 hour post-infection. The predominant products of this period are IE72 and IE86, whose expression is seen to peak between 4-8 hours post-infection. The initiation of beta (β) gene expression is ultimately dependent on the success of alpha gene expression: this begins 6-12 hours post-infection. These genes are split into two classes, β_1 and β_2 dependent upon the time of protein expression. Lastly, gamma (γ) gene expression occurs last from 24 hours post-infection, with the genes again being separated into two groups: γ_1 , the leaky late, and γ_2 , the true late genes. However, these genes are separated into two groups determined by a sensitivity to a block in viral DNA synthesis (arrow). Release of viral progeny usually occurs 96 hours post-infection.

1.6.2 Early (E) gene expression

Following expression of the MIE proteins, IE72 and IE86, a large proportion of the viral genome becomes transcriptionally active. The initiation of early (E) genes, sometimes known as the beta (β) genes, occurs from 6 hours post-infection and continues through to 24 hours post-infection when viral DNA replication initiates (see Fig. 1.5). At least 23 early genes are known to play essential roles in replication with many more having an important impact when individually disrupted in laboratory strains of HCMV (Dworsky *et al.*, 1983; Yu *et al.*, 2003). They include gene products required for viral DNA synthesis, capsid maturation and proteins necessary for altering the cellular environment and modulating the host cell response.

The early genes expressed during infection are further subdivided into two groups: the β_1 and the β_2 depending on time of expression. One set of prototypic β_1 genes is encoded by the UL112-113 region, which encodes four proteins necessary for the initiation of DNA replication. Analyses have shown that this locus is transcriptionally active during the early period of gene expression but also during the late period, with a shift in transcriptional start site occurring 62bp upstream via the ATF/CREB transcription sites and also the action of the IE86 protein (Rodems *et al.*, 1998). An example of a prototypic β_2 gene is ORF UL54, the catalytic subunit of the viral DNA polymerase. Expression of this gene is detectable by 8 hours post infection, however it reaches much higher levels at later times. Indeed, as with the UL112-113 locus, IE86 is able to cause, possibly in conjunction with the transcription factor Sp1, up-regulation of UL54 expression at later times (Wu *et al.*, 1998).

In addition to the prototypic early genes described above, a number of tegument proteins, including pp65, pp71, pp150 and the LTP are transcriptionally active as early genes even though their function is more important at maturation or upon entry into host cells. Also, intriguingly, one of the major early genes transcribed, but not translated, is the $\beta_{2.7}$ transcript (Spector, 1996). The $\beta_{2.7}$ transcript accounts for over 20% of the total RNA transcribed at this timepoint and initially investigation into its activities with HCMV strains deleted of the region found these viruses still viable in cell culture (McSharry *et al.*, 2003). However, further investigation has shown that the $\beta_{2.7}$ transcript acts to stabilise Complex I of the mitochondrion during infection, such that the production of ATP is

maintained during infection and that the mitochondrion is protected from pro-apoptotic stimuli (Reeves *et al.*, 2007).

1.6.3 Late (L) gene expression

The functions carried out by the viral late (L) genes generally involve capsid maturation, DNA encapsidation, virion maturation and egress of the virion from the cell. Despite the level of investigation into gene products expressed during this phase of infection, little is known that allows clear distinction between β_2 early genes. Many HCMV late genes are expressed with kinetics similar to those for the β_2 genes, with expression initiating within the first 24 hours post-infection. However, those being expressed after 24 hours post-infection, and also having resistance to the effects of viral DNA synthesis inhibitors, are known as the γ_1 or 'leaky' late genes. Those that are sensitive to viral DNA synthesis and its inhibitors are known as 'true' late or γ_2 genes. The expression of these genes is promoted by both viral and cellular factors (Depto & Stenberg, 1989; Depto & Stenberg, 1992), although a number of viral late promoters appear to contain little more than a TATA box to activate gene expression (Kerry *et al.*, 1997; Wing *et al.*, 1998). The pp28 tegument protein (UL99) expression occurs as two transcripts from separate transcription start sites with the major transcription occurring as a true late gene. The pp28 promoter does not appear very responsive to transactivation and does not rely on particular upstream elements, with sequences upstream of -40 (relative to the transcription start site) being completely dispensable for transcriptional activation (Depto & Stenberg, 1992; Kerry *et al.*, 1997). In addition, as with other HSV-1 truly late transcripts, the promoter is not as tightly controlled when used outside the context of the virus in an episome or when integrated into the viral genome elsewhere (Wu *et al.*, 2001b). A similar basal promoter fragment also appears to be sufficient for the UL94 true late gene (-45 to +1 relative to the start site), thus transcriptional and translational control of these genes, although important, is still not completely understood.

1.6.4 Viral proteins and the infected cell

Viral gene products expressed throughout infection are able to modulate numerous functions in the infected cell affecting cellular gene expression, cell cycle progression and cellular behaviour. The cell responds to infection in culture by HCMV immediately with modification of cellular gene expression occurring immediately upon virus attachment

probably via viral gB, as explained above (Boldogh *et al.*, 1990; Boldogh *et al.*, 1991c; Simmen *et al.*, 2001). Analysis of global changes in gene expression in the infected cell have demonstrated that pathways analagous to the toll-like receptor (TLR) signalling, interferon (IFN) response, hormone stimulation and growth factor activation occur immediately post-infection (Mocarski & Courcelle, 2001). In cell culture models, antiviral genes and inflammatory cytokine expression are activated with either HCMV virions or soluble gB alone, hence the importance of determining a receptor for this ligand during infection, with induction of IFN-stimulated genes occurring post-entry (Boehme *et al.*, 2004; Netterwald *et al.*, 2004). The overall effect of these changes is believed, in part, to activate the NF- κ B response. Although contentious, activation of NF- κ B by the virus is thought to occur in two stages: fibroblasts respond as soon as 5 minutes after exposure to virus particles which is followed by the activation of NF- κ B gene expression by 24 hours post-infection, possibly important for the enhancement of MIE gene expression (Demeritt *et al.*, 2004; Compton & Fiere, 2006).

The stimulation of cells is then modulated post-entry of the virion via both virion associated tegument proteins and with *de novo* viral gene expression. Although pp65 is dispensable for replication (Schmolke *et al.*, 1995), it is able to down-modulate the virus-mediated IFN-like response, possibly through a reduction in the level of NF- κ B activation (Browne & Shenk, 2003). The tegument protein is also thought to be able to block interferon regulatory factor (IRF)-3 activation and translocation to the nucleus (Abate *et al.*, 2004). Expression of MIE proteins is then further able to cause a second level of modulation: IE72 forming a complex with STAT1 and STAT2 preventing the association with IRF-9 and thus preventing activation IFN-responsive promoters, whilst IE86 contributes to down-modulation in ways that are not yet fully understood (Paulus *et al.*, 2006; Taylor & Bresnahan, 2006). The MIE gene products are also important for various functions during infection and will be discussed later (see section 1.9.2). In addition, another important feature of HCMV infection is the ability of certain gene products to modulate and counteract cell death which is discussed above (see section 1.6.1).

1.6.5 HCMV and cell cycle

Upon infection, HCMV is able to subvert normal cell cycle (reviewed in: Swanton & Jones, 2001); although there appears to be no absolute consensus on the effect of HCMV

on cell cycle, a number of studies have aided the elucidation of viral effects. Many studies have investigated cell cycle modulation using human fibroblasts due to the ease of obtaining synchronised cell populations and their full permissiveness for HCMV lytic infection. These studies have led to much data showing the profound effects HCMV infection has on normal cell cycle at numerous points, with the effects of infection being partly dependent upon when in the cycle infection occurred (Salvant *et al.*, 1998). Indeed, if the cell is infected during G₀ or G₁ phase, this leads to obstruction of cellular DNA synthesis. However, if the cell is infected in S phase, viral gene expression may be delayed and cells transit through S phase and mitosis (Fortunato *et al.*, 2002; Bain & Sinclair, 2007). Hence, HCMV appears to preferentially control the entry of the cell into S phase such that viral replication is optimised. The inhibition of cellular DNA synthesis seen upon infection is likely such that the virus can produce an environment highly conducive to viral DNA synthesis with this taking place through mechanisms involving several viral proteins.

Virion components themselves are able to carry out some of the initial functions necessary to establish the correct environment: in particular the tegument protein pp71 and the tegument associated gene product of UL69 (pUL69). The phosphoprotein pp71 first translocates to the nucleus upon infection of the cell (Hensel *et al.*, 1996) and is able to transactivate the MIEP to promote the expression of IE genes (Bresnahan & Shenk, 2000), which is discussed further in section 1.9. However, it also been shown in transfection assays that pp71 is able to accelerate cell cycle progression and stimulate DNA synthesis in quiescent cells via proteosomal degradation of the retinoblastoma protein, pRb, and associated pocket family proteins p107 and p130 (Kalejta *et al.*, 2003; Kalejta & Shenk, 2003a; Kalejta & Shenk, 2003b). Cell cycle progression then occurs as a result of the loss of repression on the E2F family members which are then able to activate S phase genes (reviewed in: Bain & Sinclair, 2007). pUL69, a homologue of the HSV ICP27 protein (Winkler *et al.*, 1994; Winkler & Stamminger, 1996) is further able to cause accumulation of HCMV infected cells in the G₁ phase of the cell cycle with UL69 deletion viruses impaired for viral DNA replication and late mRNA expression (Lu & Shenk, 1999; Hayashi *et al.*, 2000). Furthermore, it is thought that pUL69 acts in conjunction with pp71 and the major IE gene products to create an early S phase like state that can be exploited by the virus. It has been shown that IE72 and IE86 are both able to target the pocket family proteins, IE72 with p107 and IE86 with pRb, thus mediating G₁ to S phase progression

(Hagemeier *et al.*, 1994; Poma *et al.*, 1996). In addition, IE72 is also thought to interact directly with E2F1 and have the ability to phosphorylate other pocket family and E2F family proteins (Margolis *et al.*, 1995; Pajovic *et al.*, 1997). IE86 has also been shown to abrogate the cdk inhibitor p21 by direct interaction further aiding cell cycle progression from the G₁ to S phase (Sinclair *et al.*, 2000).

Although the ability of HCMV to advance cell cycle into early S phase would result in a beneficial environment to replicate in, this cell cycle transition would also be conducive to cellular DNA synthesis. However, as noted above, this does not occur. Studies using transient expression of IE86 in cells have shown that it also has specific roles in inhibiting cellular DNA synthesis and that this inhibition is p53 dependent (Murphy *et al.*, 2000; Wiebusch & Hagemeier, 2001; Wiebusch *et al.*, 2003a; Song & Stinski, 2005). Further work has determined that IE86 may function by targeting the formation of pre-replication complexes (pre-RCs) and interfere with their correct assembly by blocking the loading of the mini chromosome maintenance (MCM) complex onto chromatin which is an essential process in the assembly of competent RCs (Biswas *et al.*, 2003; Wiebusch *et al.*, 2003b). Furthermore, the down-regulation of chromatin licensing and DNA replication factor 1 (Cdt1) expression and an increase in the expression of Geminin, an inhibitor of replication, during HCMV infection is likely to play a part in prevention of cellular DNA synthesis (Biswas *et al.*, 2003). Although unpublished data from our laboratory suggests that IE86 alone is unable to prevent licensing of cellular origins of replication (Bain & Sinclair, unpublished), it would appear that HCMV has developed mechanisms to alter cell cycle to ensure optimum conditions for viral but not cellular DNA replication.

1.7 HCMV interactions with the immune system

Primary infection of an immunocompetent individual with HCMV is usually asymptomatic. However, virus can be detected in the secretions of infected individuals and viral DNA in the blood for up to several months after the initial infection (Zanghellini *et al.*, 1999). The ability of HCMV to avoid clearance by the immune system for such a prolonged period is likely to result from the number of mechanisms the virus employs to avoid the host immune response. HCMV encodes a number of genes that can modulate various functions involved in the host immune response. These include down-regulation of antibody and complement responses, sequestration of β -chemokines, abrogation of

apoptotic signals as well as down-regulation of the adaptive and the innate immune responses (Loenen *et al.*, 2001; Braud *et al.*, 2002; Mocarski, 2002). These mechanisms allow the virus to evade clearance and enable viral persistence and latency.

1.7.1 HCMV and the innate immune system

The innate immune system is devoted to sensing and controlling microbial replication during the time that precedes the induction of the adaptive immune response. The cells of the innate immune system use receptors that recognise structurally conserved molecular patterns on a range of microorganisms, including viruses, with major receptors including Toll-like receptors (TLRs) that recognise pathogen-associated molecular patterns (PAMPs). However, cells of the immune system are also able to recognise certain endogenous ligands with the importance of the NKG2D receptor and ligands during virus infection becoming apparent in recent times.

The immune cells to come into contact with virus and virus infected cells first appear to be the Natural Killer (NK) cells. NK cells are clearly important for control of HCMV infection and consistent with this HCMV encodes a number of genes to inhibit NK-mediated killing of infected cells (Mocarski, 2004). In addition, it has also been demonstrated that MHC class I down-modulation by HCMV makes cells more susceptible to NK cell lysis (Falk *et al.*, 2002). A number of viral MHC mimics and other MHC-interacting proteins have been demonstrated and shown to be inhibitors of NK cell activity, such that the infected cells are not killed due to loss of MHC class I expression on the cell surface as has been understood for some time (Ljunggren & Karre, 1985). These include the viral UL16, UL18, UL40, UL141 and UL142 gene products that all play roles in NK cell down-modulation via varying mechanisms (see Fig 1.6) (reviewed in: Wilkinson *et al.*, 2008). The UL16 protein, first found as a glycoprotein dispensable for growth *in vitro* (Kaye *et al.*, 1992), was later found to down-regulate activating ligands for NKG2D (ULBP1, ULBP2, and MICB) (Wilkinson *et al.*, 2008) thus inhibiting cell lysis, whereas the UL40 gene product binds to HLA antigen-E to maintain surface expression of inhibitory ligands and retain NK repression via CD94:NKG2A (Tomasec *et al.*, 2000). Additionally, UL18 is a virally encoded MHC class I homologue and expression on the cell surface further inhibits NK cell mediated lysis of infected cells (Browne *et al.*, 1992; Leong *et al.*, 1998).

Recently it has become clear that the UL_b' region of the viral genome is dispensable for *in vitro* growth of HCMV but is found in clinical isolates. Examination of the proteins encoded by this region has brought further NK cell evasion tactics to our attention. The UL141 protein post-translationally down-regulates the NK cell activatory ligand CD155 which is recognised by the activating receptor CD226 (Tomasec *et al.*, 2005). Additionally, the UL142 protein has been identified as having MHC class I homology that inhibits NK cell lysis in a clonally dependent manner (Wills *et al.*, 2005) and that this may be mediated by down-regulation of MICA, an activating ligand for NKG2D (Chalupny *et al.*, 2006). However, UL142 cannot target all alleles of the MICA molecule known in the human population. Consequently, UL142 may target multiple NK cell ligands. Other mechanisms the virus has developed to target ligands for NKG2D include the recently highlighted micro RNA (miRNA) pathway. HCMV is able to abrogate the expression of a further ligand, MICB, through production of the miR-UL122-1 miRNA (Stern-Ginossar *et al.*, 2007). Therefore, the importance of the NK cell immune response is obvious with the number of overlapping methods employed by HCMV to inhibit this defence.

1.7.2 HCMV and the adaptive immune system

As with modulation of the innate immune response, HCMV encodes a number of genes that target the adaptive immune response (see Fig. 1.6). The US2-US11 region has been shown to be important for regulation of immunosurveillance by targeting the antigen presentation pathway in multiple ways. The CD8⁺ cytotoxic T lymphocyte (CTL) response is abrogated by the down-regulation of antigen presentation via retention of MHC class I in the ER by the US3 gene product (Ahn *et al.*, 1996), destruction of MHC class I molecules by the US2 and US11 proteins (Hengel *et al.*, 1996) and inhibition of peptide loading onto MHC class I by prevention of TAP-mediated translocation of peptides across the ER by US6 (Lehner *et al.*, 1997). Additionally, HCMV also phosphorylates the major antigen IE72, mediated by pp65, such that it can no longer be processed for antigen presentation to T cells (Gilbert *et al.*, 1996). Furthermore, HCMV also targets the CD4⁺ T cell response by down-regulating the expression of MHC class II molecules: this occurs via the US2 gene product (Tomazin *et al.*, 1999; Johnson & Hegde, 2002). The virus also encodes a viral homologue of IL-10 that may down-regulate MHC class II expression on the cell surface, with evidence of transcription during latency and productive infection.

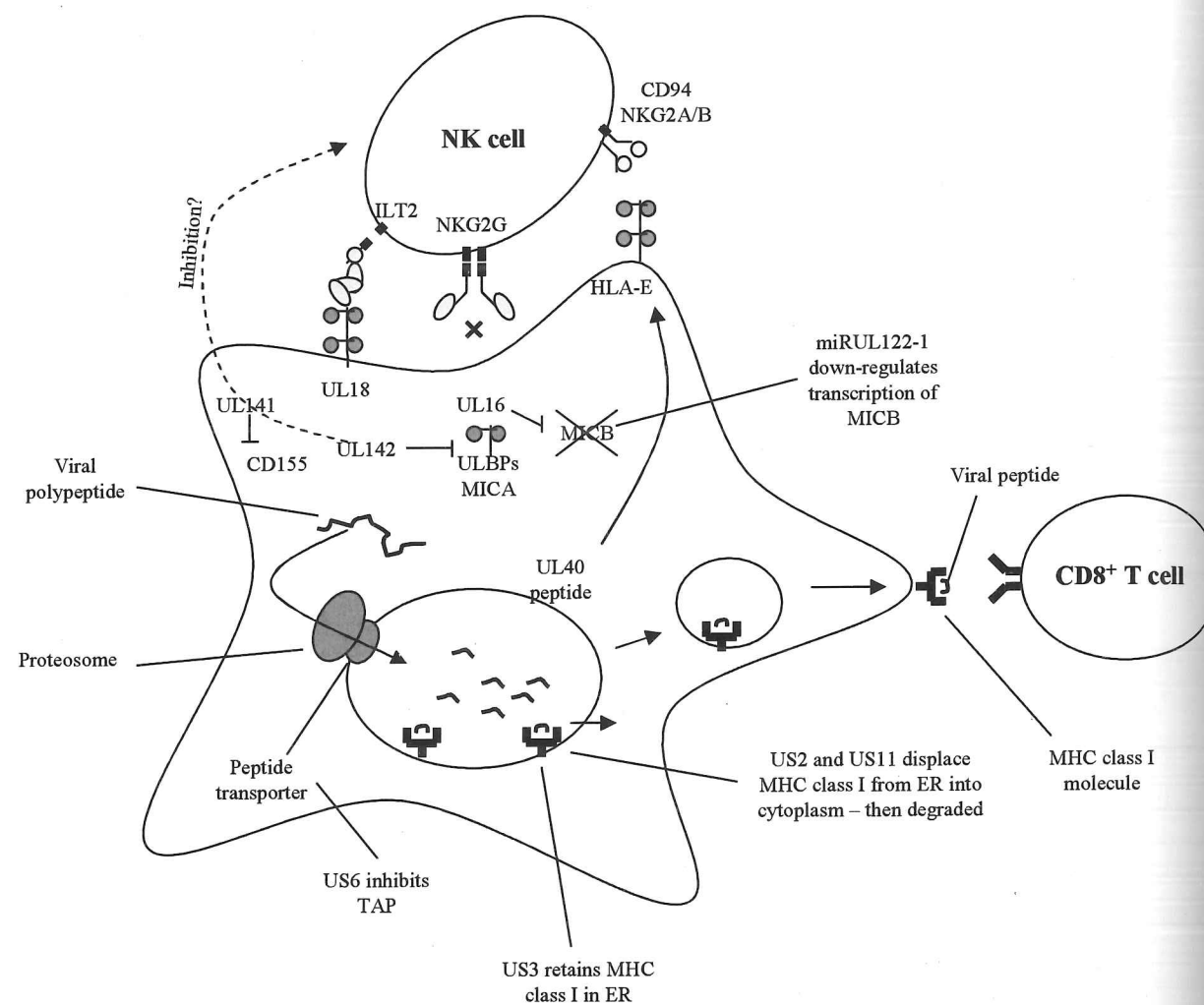


Figure 1.6 CD8⁺ T cell and NK cell evasion by HCMV infected cells. During HCMV infection, the viral proteins US2, US3, US6 and US11 cause the surface expression of MHC class I molecules to progressively diminish, disrupting processing and presentation of antigen peptide to CD8⁺ T cells. This could lead to NK cell recognition of the infected cell and thus the virus encodes a further set of proteins UL16, UL18, UL40, UL141 and UL142, and a miRNA (miRUL122-1), to further modulate the expression of surface markers and inhibit NK cell activation and infected cell lysis. (Adapted from Wills *et al.*, 2006).

(Jenkins *et al.*, 2004; Jenkins *et al.*, 2008), although there are no data to confirm the importance of this homologue during productive viral infection or latency (Kotenko *et al.*, 2000; Pepperl-Klindworth *et al.*, 2006).

Despite the numerous virus-encoded functions to evade the immune response, HCMV infection is generally well controlled in immunocompetent hosts. An analysis of the T cell antigen specificities in the memory pool has indicated that more than 1% of the CTLs recognise HCMV epitopes. Although work has demonstrated that this pool can be specific for a number of HCMV glycoproteins, tegument proteins and others (Boppa & Britt, 1996), it is generally accepted that the major epitopes recognised are from the pp65 and IE72 proteins (McLaughlin-Taylor *et al.*, 1994; Wills *et al.*, 1996; Gillespie *et al.*, 2000). This prevalence of HCMV-specific CTLs is likely due to the duration of primary infection but also probably an indication of the exposure to viral antigen upon sporadic sub-clinical reactivation of the virus.

1.7.3 HCMV additional immune evasion tactics

A further threat to HCMV infected cells is complement mediated lysis. One study has shown that the virus is able to incorporate cellular proteins that are important for complement binding to cells, CD55 and CD59, into virions (Spear *et al.*, 1995). It is thought that delivery of these proteins into the cell upon infection could aid down-regulation of complement and thus prevent the process leading to cell lysis. Therefore, it isn't surprising that HCMV has been shown to up-regulate the expression of complement regulators such as CD55 and CD46 on the cell surface following infection to further promote an environment that prevents the action of complement (Spiller *et al.*, 1996). The virus is also able to affect multiple signalling pathways between infected cells and effector cells of the immune system. Cells are able to respond to extracellular cytokines via the action of G-protein coupled receptors (GPCRs) that inactivate a number of cellular signalling pathways. HCMV encodes four GPCR homologues (Chee *et al.*, 1990b) of which US28 appears functionally active, encoding a C-C chemokine GPCR (Neote *et al.*, 1993) capable of binding CC (Gao & Murphy, 1994) and CX3C chemokines (Kledal *et al.*, 1998). The outcome of US28 expression is likely to be the alteration of the infected cells to extracellular chemokines via sequestration such as those promoting chemotaxis (Bodaghi *et al.*, 1998), however more recent data confounds the importance of US28 altogether

(Hertel & Mocarski, 2004). Chemokines likely to be affected are 'regulated on activation, normal T cell expressed and secreted' (RANTES) and macrophage inflammatory protein-1 β (MIP-1 β), as following infection of endothelial cells (ECs), RANTES secretion is reduced. This could possibly assist evasion of local immune responses (Billstrom Schroeder & Worthen, 2001). US28 has also been proposed to promote smooth muscle cell (SMC) migration, which may be the result of intracellular signalling cascades activated upon binding to RANTES (Strebblow *et al.*, 2003). Viral chemokines encoded by HCMV also have a role during infection: the UL146 gene product encodes a potent alpha chemokine, vCXC-1, which is an IL-8 homologue (Penfold *et al.*, 1999) that attracts neutrophils and possibly myeloid cells expressing CXCR2 to the site of infection. It is possible that the biological role of this process is to attract cells that may be able to disseminate the virus to other sites in the host, including cells known to be sites of latency. The importance of this type of process has gained further weight with a study suggesting that HCMV infection may promote monocyte differentiation and migration as a vehicle for dissemination (Smith *et al.*, 2004), although other studies have suggested that HCMV infection inhibited differentiation, perhaps aiding the formation of latency (Gredmark & Soderberg-Naucler, 2003).

1.8 Latency and reactivation

Latency, or the ability of a virus to establish a lifelong persistent infection of a host in the absence of any detectable production of infectious virus, is a property of HCMV that is common to all the herpesviruses (reviewed in: Sinclair & Sissons, 2006; Sinclair, 2008). Establishment of latency occurs following primary infection and, although a hallmark of the *herpesviridae*, is not exclusive to this family. For instance other viruses such as the retroviruses also appear to persist as a latent infection. Latency is likely to be broken by sporadic reactivation events, ultimately facilitating dissemination, and normally only leading to either sub-clinical or benign clinical symptoms. However, reactivation of herpesviruses can pose a serious health threat in patients who are severely immunocompromised or immunosuppressed, particularly in the case of HCMV, and thus a number of studies have been undertaken to determine the mechanisms that control this event.

1.8.1 Sites of human herpesvirus latency

In order to understand HCMV latency, it is first important to establish where or in which cell types the virus is latent. Sites of latency of human herpesviruses differ between the sub-families and species of each. The alphaherpesviruses HSV-1 and HSV-2 are neurotropic. After primary replication at the site of infection, HSV-1 is able to gain access to sensory nerve terminals and, following retrograde axonal transport to neuronal cell bodies, is able to establish latency within trigeminal ganglia. In the case of HSV-2, which is usually sexually transmitted, this occurs within neurons of the anatomically related sacral ganglia (Efsthathiou & Preston, 2005).

The gammaherpesvirus Epstein-Barr virus (EBV), a member of the *Lymphocryptovirinae*, is restricted to the B lymphocyte population of the lymphoid cell lineage. Infection of resting B cells results in their conversion to a lymphoblastoid cell which can be propagated as a lymphoblastoid cell line (LCL) (Aman *et al.*, 1994; Aman *et al.*, 1996) and defines the oncogenic potential of the virus. Similarly, another gammaherpesvirus Kaposi's Sarcoma-associated herpesvirus (KSHV) is also able to establish a persistent infection of latently infected B cells (Decker *et al.*, 1996). In addition, like EBV, infection of B cells with KSHV causes transformation to a LCL phenotype (Kliche *et al.*, 1998).

Similar to the other sub-families of the herpesvirus family, betaherpesviruses share a number of similarities in growth kinetics, genomic structure and coding and indeed also share sites of latency. The DNA of the *Roseoloviruses* HHV-6A and HHV-6B can be detected in the peripheral blood mononuclear cells (PBMC) of healthy seropositive individuals (Cuende *et al.*, 1994) as is the case with HCMV (Taylor-Wiedeman *et al.*, 1991). However, both HHV-6 sub-species and HHV-7 additionally show a preferential tropism for T lymphocytes (Ablashi *et al.*, 1991), whereas in contrast the presence of HCMV DNA in T lymphocytes cannot be detected (Taylor-Wiedeman *et al.*, 1993).

1.8.2 HCMV latency and the myeloid lineage

Healthy HCMV-seropositive blood donors are able to transmit HCMV infection and transmission of HCMV in donated blood can be reduced by using leukocyte depleted blood products. Consequently, one site of carriage of the virus has long been proposed to be in the peripheral blood compartment (Yeager *et al.*, 1981; Adler, 1983; Tolpin *et al.*, 1985; de

Graan-Hentzen *et al.*, 1989). However, identification of the blood component carrying viral genome only occurred with the advent of sensitive analysis techniques such as polymerase chain reaction (PCR) allowing detection of low copy numbers of viral DNA sequences viral DNA. Using this approach, many groups have been able to show the presence of HCMV DNA in the peripheral blood leukocytes of healthy seropositive individuals (Taylor-Wiedeman *et al.*, 1991; Stanier *et al.*, 1992; Larsson *et al.*, 1998). Further analysis, using granulocyte-macrophage colony-stimulating factor (GM-CSF)-mobilised peripheral blood cells has shown that the frequency of mononuclear cells carrying viral genome could be as low as 1:10,000 (Slobedman & Mocarski, 1999).

By sorting PBMC, it has now been shown a major site of carriage of the virus is the peripheral blood monocyte population (Taylor-Wiedeman *et al.*, 1991; Larsson *et al.*, 1998). Monocytes arise from pluripotent CD34⁺ stem cells present in bone marrow, which are able to differentiate along the myeloid lineage to monoblasts, then promonocytes in the bone marrow before entering the blood stream where they lose the CD34 surface antigen and develop into monocytes (Katz *et al.*, 1985). Interestingly, the presence of HCMV DNA can also be detected in these CD34⁺ bone marrow progenitors (Mendelson *et al.*, 1996). Interestingly, these progenitor cells are also able to develop into B cells, T cells and polymorphonuclear leukocytes (PMNL). However, HCMV genome has never been detected in these cells (see Fig. 1.7) (Taylor-Wiedeman *et al.*, 1991; Taylor-Wiedeman *et al.*, 1993). Analyses using reverse transcription (RT)-PCR with the aim of detecting viral lytic transcripts in CD34⁺ cells and myeloid derivatives of healthy, seropositive carriers has also failed to detect viral IE gene expression despite the presence of HCMV genome in these cells (Taylor-Wiedeman *et al.*, 1994; Mendelson *et al.*, 1996), consistent with a lack of any virus production. However, differentiation with hydrocortisone (HC) or phorbol myristate acetate (PMA) to a macrophage-like cell was sufficient to initiate lytic gene expression (Taylor-Wiedeman *et al.*, 1994) consistent with the finding that macrophages are permissive for HCMV infection *in vitro* (Ibanez *et al.*, 1991).

Although there is now good evidence that myeloid cells are a true site of HCMV latency *in vivo*, it is also possible that other sites of latent virus may exist in a normal healthy carrier. CD34⁺ cells, as well as giving rise to the myeloid lineage, may also be able to develop into endothelial cells (EC) (Quirici *et al.*, 2001) and therefore the possibility of this cell pool

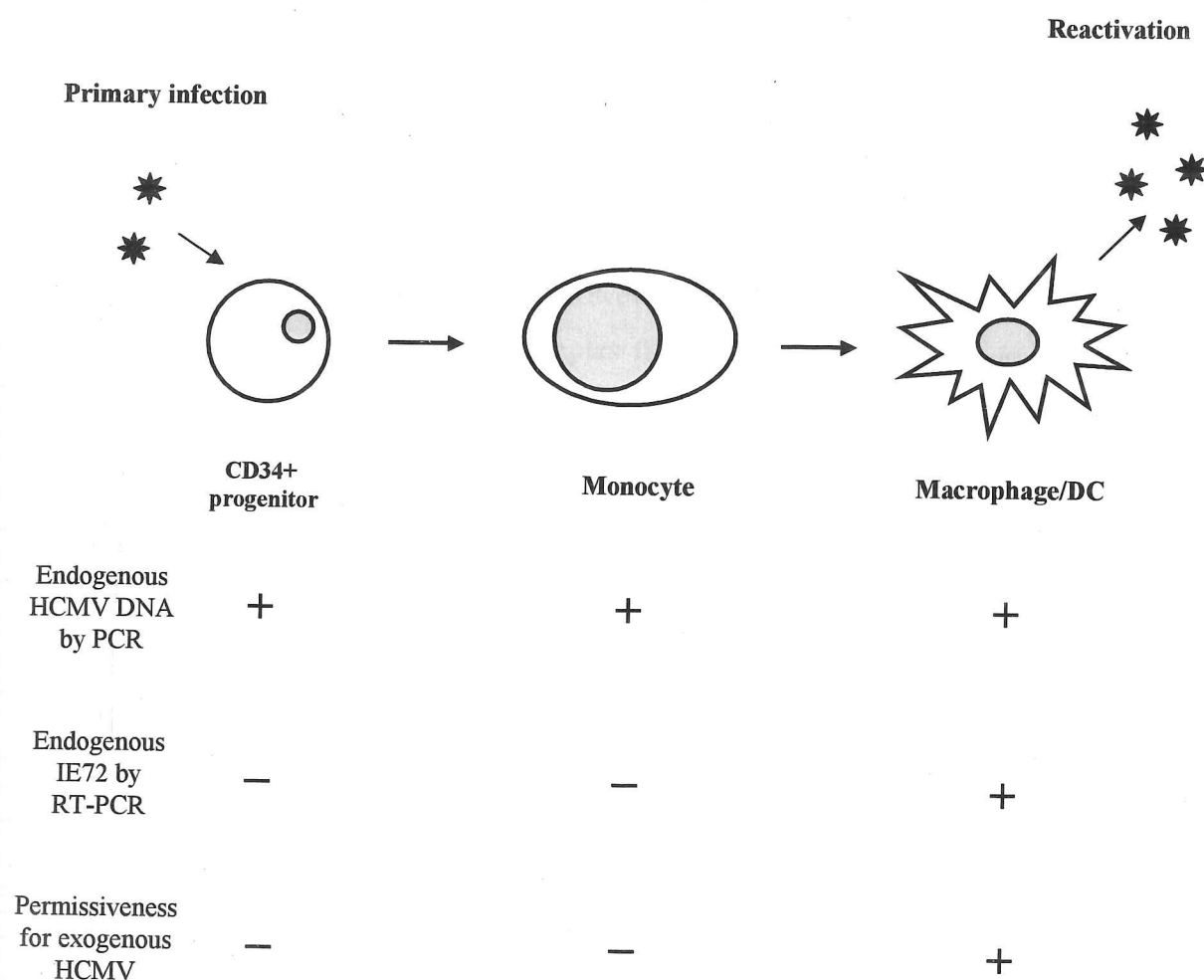


Figure 1.7 Model of HCMV latency and reactivation. Viral DNA can be detected in all cells of the myeloid lineage during latent carriage of a normal seropositive HCMV carrier. However, viral IE gene expression only occurs upon differentiation of cells (macrophage/DC) due to a change in transcriptional milieu conducive to MIEP activation and reactivation. (Adapted from Sinclair & Sissons, 2006).

acting as a reservoir for latent virus has been proposed (Jarvis & Nelson, 2002). However, despite circumstantial evidence that has linked HCMV with atherosclerosis (Epstein *et al.*, 1996), work from our laboratory has been unable to show any presence of latent viral DNA in ECs or vascular smooth muscle cells from a seropositive individual even though latent DNA could be readily detected in monocytes concomitantly (Reeves *et al.*, 2004). Further work, addressing the possibility of persistence via low-level productive infection in aortic ECs acting as a mechanism for what appears to be latent carriage has been shown (Fish *et al.*, 1996; Streblow & Nelson, 2003). However, due to the specific tissue in question, it remains difficult to obtain *ex vivo* samples from healthy seropositives to confirm these cells as sites of HCMV latency *in vivo*.

Since the carriage of HCMV genome in myeloid cells *in vivo* is not associated with any substantial level of lytic infection (Taylor-Wiedeman *et al.*, 1994; Mendelson *et al.*, 1996), it is unlikely that genome persists due to low-level productive infection in these cells. Some evidence does support the existence of the viral genome in an episomal formation during carriage in monocytic cells (Bolovan-Fritts *et al.*, 1999) suggesting that the genome could be maintained in a similar fashion to the EBV genome during latency. In EBV, the latent gene product Epstein-Barr nuclear antigen 1 (EBNA1) tethers the episomal viral DNA to the cellular chromosomal DNA (Adams, 1987; Leight & Sugden, 2000). However, there is little evidence for a latent origin of replication in HCMV, although deletion analyses near to the MIE region has defined a region of the HCMV genome that may be involved in genome maintenance during experimental latency in granulocyte-macrophage precursors (GMPs) (Mocarski *et al.*, 2006). Consequently, the mechanism by which the HCMV genome persists in the absence of lytic infection in cells of the myeloid lineage remains largely enigmatic.

1.8.3 Maintenance of herpesvirus latency

The mechanism by which herpesvirus latency is maintained has been much investigated and studies performed across the herpesvirus families have identified a number of transcripts produced from viral genomes during latent infection. Most notable was the identification of a latency-specific, large non-coding RNA known as the latency associated transcript (LAT) in HSV-1 (reviewed in: Preston, 2000). The LAT was first identified as a mRNA species that was anti-sense to the alpha genes and was expressed during HSV-1

latent infection of neurons (Stevens *et al.*, 1987). Further work determined that the LAT was an 8.5-9kb product that was inherently unstable and was also spliced to produce a further major 2.2kb product (Wagner *et al.*, 1988), although current understanding is that poly A negative transcripts of 2.0 and 1.5 kb termed major LATs are derived by splicing of the 8.3kb primary transcript termed minor LAT (mLAT) (Efsthathiou & Preston, 2005). The LAT has been found to regulate the establishment of latency (Thompson & Sawtell, 1997) and a number of mechanisms for LAT function during latency have been proposed, including the anti-sense down-regulation of ICP0 and ICP4, modification of the genome, control of reactivation and active retention of the virus in the latent state (reviewed in: Roizman *et al.*, 2007). Some studies have shown that the LAT actively prevents IE gene expression (ICP0, ICP4 and ICP27) thus promoting latency (Mador *et al.*, 1998). This supports earlier work where studies using LAT null mutants demonstrated an increase in the overall expression of lytic genes (Garber *et al.*, 1997) which was concomitant with a reduction in the number of neurons in which latent infection was established. However, other work has suggested the necessity of the first 1.5kb of the LAT for reactivation (Perng *et al.*, 1996) and, additionally, that the LAT is responsible for preventing apoptosis and promoting survival of neurons during infection with HSV (Inman *et al.*, 2001; Thompson & Sawtell, 2001). Differing opinion still exists as to the action of the LAT during productive infection, although it is agreed that this region remains important during latent infection.

In contrast to HSV, EBV encodes a number of proteins that are expressed during latent infection of B lymphocytes (reviewed in: Young & Rickinson, 2004). These latent proteins comprise six EBV-encoded nuclear antigens, EBNA1, 2, 3A, 3B, 3C and -LP, and three latent membrane proteins, LMP1, 2A and 2B. In addition, LCLs also show expression of small, non-polyadenylated RNAs termed EBER1 and EBER2, although the function of these transcripts is still yet to be fully determined (Middleton *et al.*, 1991). The expression of these genes can differ depending on the stage of infection and differentiation state of the infected cell. These differing variations of latent gene expression have been found specific to certain malignancies: type I latency with Burkitts lymphoma and type II latency with Hodgkins disease (Rickinson & Kieff, 2007). However, the full pattern of gene expression described above is referred to as latency III causing proliferation of B cells whilst latency 0/I and II only occur in resting B cells.

EBNA1, as well as being found to interact with promoters of other EBNAs and influence transcriptional regulation, is able to bind to the plasmid origin of replication (OriP) and maintain the episomal viral genome in the transformed cells (Sugden & Warren, 1989). EBNA1 elicits only a poor CTL immune response due to a Gly-Ala repeat in the protein that limits proteasomal breakdown and its presentation on the cell surface to CTLs (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). The EBNA2 gene product is crucial for the establishment of transformation of cells. It appears to function by interacting with a sequence-specific DNA binding protein, J κ -recombination-binding protein (RBP-J κ), to activate transcription of cellular genes such as CD23 and the viral LMP1 and LMP2A genes (Grossman *et al.*, 1994; Hsieh & Hayward, 1995). Additionally, EBNA2 is able to interact with EBNA-LP which is necessary for efficient outgrowth of virus-transformed cells *in vitro* (Mannick *et al.*, 1991; Sinclair *et al.*, 1994). The transcriptional activation driven principally by EBNA2 but also EBNA-LP, LMP1 and LMP2A is directed by the EBNA3 family: EBNA3A-C are able to repress transactivation (Robertson *et al.*, 1996), most likely through interactions of the proteins with transcriptional modulators (Radkov *et al.*, 1999).

The LMP1 protein is essential for transformation of infected B cells (Wang *et al.*, 1985; Kaye *et al.*, 1993). LMP1 has many effects on the cells from inducing the production of cell surface adhesion molecules and an up-regulation of anti-apoptotic genes (Eliopoulos & Young, 2001). Resembling CD40, providing both growth and differentiation signals to the infected B cells and acting as a constitutively activated tumour necrosis factor receptor (TNFR), LMP1 activates several downstream signalling pathways in a ligand-independent manner (Mosialos *et al.*, 1995; Eliopoulos & Young, 2001). In contrast, the LMP2 proteins (LMP2A and LMP2B) are not essential for B cell transformation *in vitro* (Longnecker, 2000). However, LMP2A is able to drive proliferation and survival of the B cell in the absence of signalling from the B cell receptor (BCR) and can transform epithelial cells and increase their adhesion and motility (Scholle *et al.*, 2000). The LMP2A protein acts to induce gene expression of cellular genes involved in cell-cycle induction, inhibition of apoptosis and suppression of cell-mediated immunity which likely provides an anti-differentiation environment to enhance cell survival (Longnecker, 2000). The result of all these virus directed changes to cellular signalling and gene expression ultimately cause the

infected cell to take on characteristics of a memory B cell to aid long term maintenance of the viral genome.

The betaherpesviruses have also been found to encode latency associated transcripts. HHV-6 appears to encode a single latency transcript in the PBMCs of seropositive hosts (Rotola *et al.*, 1998; Kondo *et al.*, 2002). Transcripts from across the U94 gene locus have been detected and although their exact function remains unclear, they appear to promote the establishment of latency through inhibition of viral lytic gene expression (Rotola *et al.*, 1998). In contrast, no latent transcription has been found associated with HHV-7 infection of PBMC.

Latency associated transcripts have also been analysed during HCMV infection. Using experimental latency models of GMPs, several transcripts have been identified that are expressed in the absence of virus production in long term culture (Kondo *et al.*, 1994; Kondo & Mocarski, 1995). These transcripts have been termed cytomegalovirus latency-specific transcripts (CLTs) and include novel spliced and unspliced transcripts from both strands of the HCMV MIE region of the genome. Some of these transcripts have also been identified in healthy, seropositive carriers of the virus (Kondo & Mocarski, 1995; Kondo *et al.*, 1996; Hahn *et al.*, 1998) as well as in infected cells in culture (Lunetta & Wiedeman, 2000). Antibodies to some putative CLT ORFs have also been detected in healthy carriers (Landini *et al.*, 2000). However, no viral gene products from any of these regions have, so far, been confirmed to play a role during viral latency. Using the same experimental system, a novel transcript from the UL111.5A region of the genome has also been identified that is predicted to encode a viral homologue of IL-10 (vIL-10) with the suggestion that expression during latency may aid avoidance of the immune system. However, this transcript has also been detected in monocytes from HCMV seronegative individuals (Jenkins *et al.*, 2004) and therefore its importance remains somewhat unclear.

Long term growth of CD34⁺ progenitor cells from healthy seropositive individuals has also been used to further examine the possibility of viral transcription and gene expression during latency (Goodrum *et al.*, 2002). Initial work identified a number of viral RNAs associated with carriage of the virus in the absence of the production of viral progeny. However, some transcripts detected were known lytic cycle genes (Goodrum *et al.*, 2002).

Further analysis of the sub-populations of these cells showed that different sub-populations were able to support HCMV infection to different levels, ranging from productive infection to latency (Goodrum *et al.*, 2004). Consequently, whether these transcripts identified truly represent latency associated transcription awaits further analysis. However, based on this work, analysis of RNA isolated from monocytes of healthy seropositives has identified viral transcripts anti-sense to the UL81-82 region of the viral genome from which the MIEP transactivator phosphoprotein pp71 is encoded (Bego *et al.*, 2005). This led to the suggestion that the gene product could be involved in the maintenance of latency by restricting the induction of IE gene expression (Bego *et al.*, 2005), although there is no direct evidence for this. Furthermore, an additional transcript identified during experimental infection of hematopoietic progenitor cells *in vitro* and confirmed *in vivo* is produced from UL138, an ORF of the UL_b' region, and may help to establish or maintain latency during natural infection (Goodrum *et al.*, 2007).

1.8.4 Herpesvirus reactivation

Reactivation from latency is dependent upon the initiation of lytic gene expression from endogenous virus genomes which necessarily must occur in the absence of transactivating virion structural proteins. Reactivation has been linked to a number of scenarios, including host stress and immunosuppression.

Many studies on HSV have provided evidence that the exit of the virus from a latent state is dependent on the activation and expression of ICP0 (Wilcox *et al.*, 1997). Infection studies with ICP0 null mutants at a low multiplicity of infection (MOI) have shown a predisposition of the viruses to a latent state (Stow & Stow, 1986; Sacks & Schaffer, 1987) and these mutants also show greatly reduced ability to reactivate from latency in animal models (Leib *et al.*, 1989; Halford & Schaffer, 2001). In addition, it has also been shown that ICP0 expression is capable of aiding reactivation of the latent genome (Harris *et al.*, 1989; Zhu *et al.*, 1990; Preston & Nicholl, 1997). Although, in these experiments the differences between the ICPO null mutants used and the methods of quantifying the efficiency of reactivation does make it difficult to determine how important ICP0 is during this transition. Furthermore, additional analyses have focussed on the possibility of reactivation being a consequence of the number of virus genomes present per infected cell: work with infected mice demonstrated a correlation of reactivation with the number of

latently infected neurones in the ganglia (Sawtell, 1998) whilst infection of permissive cells with ICP0-null mutants illustrated a limit of the efficiency of reactivation at low MOIs and that competition between genomes occurs at high MOIs (Preston, 2007). However, it remains that most models of HSV reactivation focus on either the activation of the ICP0 promoter and subsequent ICP0 expression through induced cellular factors or the induction of cellular proteins that imitate the action of ICP0 (Cai & Schaffer, 1991; Yao & Schaffer, 1995; Jordan *et al.*, 1998; Loiacono *et al.*, 2003). The expression of ICP0, which is a promiscuous activator of viral gene expression and functions as a DNA sequence-independent transcription factor (Efsthathiou & Preston, 2005), leads to the activation of many promoters most likely through a RING finger domain that mediates E3 ubiquitin ligase action (Everett *et al.*, 1998b). Subsequently, targeted ubiquitination leads to the proteasomal degradation of a number of cellular proteins, including components of ND10 (for further discussion see section 1.11). Another feature of ICP0 that may aid its role as a mediator of reactivation is the ability of the protein to interact with a number of other cellular and viral proteins (Hagglund & Roizman, 2004). One such viral protein is ICP4. Some studies have suggested that co-activation of ICP0 and ICP4 could be sufficient for the initiation of reactivation (Yang *et al.*, 2002). Therefore, although the data are not completely equivocal either *in vitro* or *in vivo* analysis, it appears that the reactivation of HSV is likely to be dependent on the activation of IE gene expression but this still remains in need of further examination.

The reactivation process of the gammaherpesviruses is exemplified by EBV. As with HSV, although the process is still not fully understood, it is likely to involve the down-regulation of latency associated viral proteins and the reactivation of lytic gene expression. Reactivation of EBV from Burkitts lymphoma cell lines can be induced by cross-linking of surface immunoglobulins or by the addition of TGF β (Takada & Ono, 1989; di Renzo *et al.*, 1994). The exact consequences of these effectors are unknown, although it is likely that they cause a change to the transcriptional milieu of the cell which is conducive for expression of the master regulator of EBV lytic activation, BZLF1. Furthermore, specific elements (Zp) have been found in the promoter of BZLF1 which are necessary for reactivation of BZLF1 expression in response to BCR cross-linking (Binne *et al.*, 2002). In addition, recent work has identified other transcription factors that are involved in the reactivation of BZLF1 expression in certain latent cell types: in plasma cell lines and other

lymphoid lines, the plasma cell differentiation factor XBP1 has been shown to activate expression of IE genes (Sun & Thorley-Lawson, 2007). Also, a further cellular transcription factor ZEB1 has also been found recently to mediate reactivation, although in infected 293 cells (Yu *et al.*, 2007b). Indeed, it is now thought that changes in the chromatin structure of the BZLF1 promoter is critical for regulation of latency and reactivation (Bryant & Farrell, 2002), which may be mediated by differentiation-dependent transcription factors. It is becoming increasingly clear that reactivation of EBV may mechanistically be similar to that of HCMV.

The clear link between the process of differentiation of a myeloid cell infected with HCMV and reactivation of IE gene expression (Taylor-Wiedeman *et al.*, 1994) and production of infectious virus (Soderberg-Naucler *et al.*, 1997) has led to the prediction that HCMV reactivation is ultimately dependent upon myeloid cell differentiation. A number of model cell lines predict that HCMV latency may involve repression of IE gene expression upon infection through the action of transcription factors which repress the viral MIEP present specifically in undifferentiated cells. However, upon differentiation, the transcriptional milieu of the cell alters and an environment that is conducive for IE gene expression occurs (Sinclair & Sissons, 2006). Unfortunately, the lack of animal models for HCMV and the lack of tractable *in vitro* model systems for HCMV latency have made analysis to determine the factors which control HCMV reactivation difficult.

Although there are few good model systems in which to systematically analyse factors controlling HCMV latency and reactivation *in vitro*, certain cell systems that recapitulate the differentiation-dependent regulation of viral IE gene expression have been useful for these types of analyses. Certain cell models have led to an understanding of the role of cellular transcriptional activators and repressors of viral gene expression in latency and reactivation (Meier & Stinski, 1996). The embryonal carcinoma cell system NT2D1 (T2 cells) and the myelocytic myeloma cell line THP1 are non-permissive for viral infection due to a block in major IE gene expression. However, their differentiation results in a fully permissive phenotype. Both these cell lines have been used extensively to determine cellular factors important for permissiveness of HCMV infection and will be further discussed in Chapter 5. In general, however, overall it has been shown that decreases in transcriptional repressors concomitant with increases in transcriptional activators of viral

IE gene expression dictate permissiveness for HCMV IE gene expression and that this is mediated by chromatin remodelling of the MIEP (Murphy *et al.*, 2002).

1.9 HCMV major immediate early region

As alluded to earlier, whether the outcome of HCMV infection is lytic or latent appears to be determined by whether or not viral IE gene expression is supported in the infected cell. The commencement of a fully lytic cycle is absolutely determined by whether transcriptional activation of MIE gene expression occurs and this depends on whether the major immediate early promoter (MIEP) is activated or repressed.

1.9.1 HCMV major immediate early promoter (MIEP)

The HCMV MIEP, sometimes termed the MIE promoter/enhancer, is one of the strongest known transcriptional promoters. The MIEP is composed of a number of different regions: a core promoter; an enhancer region, split into proximal and distal areas; a unique region; and a modulator area (see Fig. 1.8). Although the regulation of the MIEP is not fully understood, much information has been gained by transfection analyses of MIEP reporter constructs. Such analyses have shown that the transcriptional activity of the MIEP varies according to a number of criteria, including cell type, differentiation status and the activity of certain signalling pathways (reviewed in: Meier & Stinski, 1996). Overall, it has been determined that the conditional activity of the MIEP results from a range of cellular and viral factors that are able to both positively and negatively regulate MIEP transcriptional activity.

The enhancer region of the HCMV MIEP is around 540bp in length and the two halves of the enhancer, distal and proximal, appear structurally divergent but function together to cause efficient promoter activation (Meier & Pruessner, 2000; Isomura & Stinski, 2003). It has been suggested that the distal element of the enhancer is important for interaction with virion components during lytic infection, as removal of this region has been found to significantly decrease MIEP activity at low MOIs but this can be overcome with infection at higher MOIs (Meier & Pruessner, 2000; Meier *et al.*, 2002). Indeed, the presence of multiple *cis*-acting elements in the distal enhancer has been shown to determine promoter activity (Meier *et al.*, 2002). Experiments using truncation analysis of the proximal end have also demonstrated further positive *cis*-acting elements due to the progressive loss of

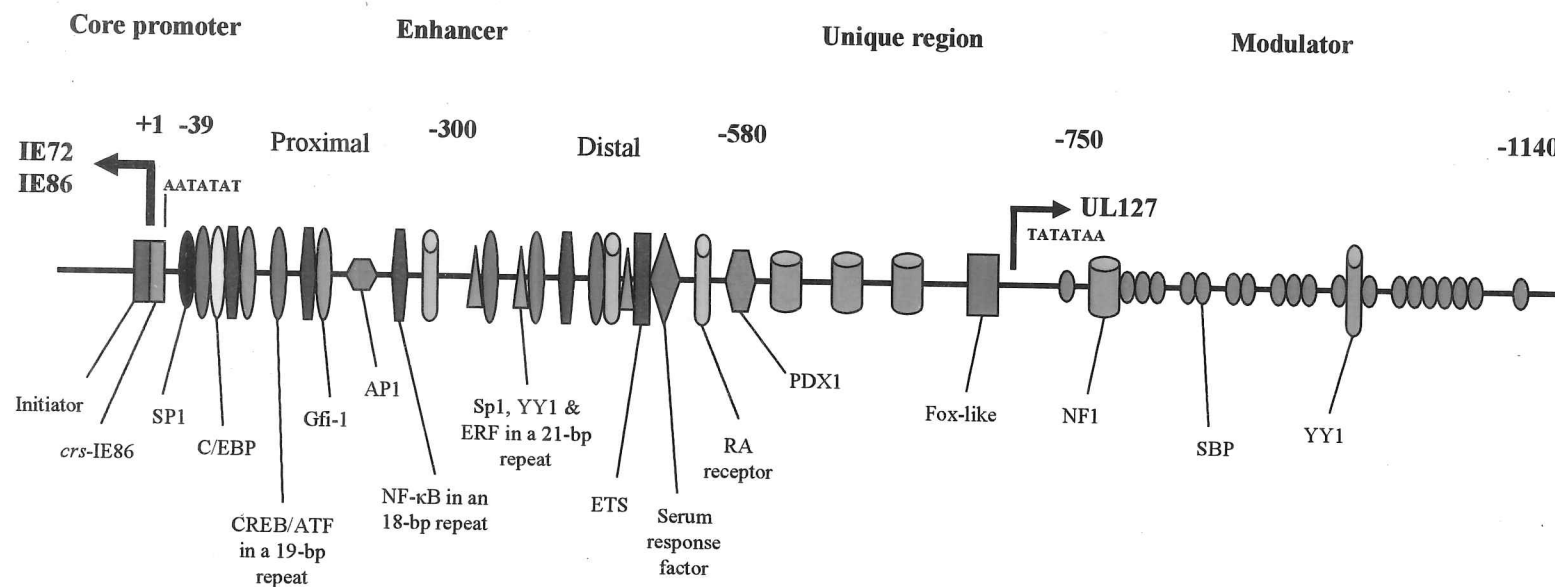


Figure 1.8 The HCMV MIE promoter/enhancer (MIEP). The MIEP is composed of a core promoter, an enhancer, a unique region and a modulator. In addition, the enhancer is divided into proximal (-39 to -299) and distal (-300 to -579) portions. The positions of putative binding sites for the initiator complex, C/EBP, CREB/ATF, Gfi-1, AP1, NF- κ B, Sp1, YY1, ERF, ETS, serum response factor, retinoic acid (RA) receptor, PDX1, NF1 and SBP are shown relative to the +1 transcription start site of the core promoter. The binding sites for NF- κ B, CREB/ATF and the combination of SP1, YY1 and ERF are nested in the 18, 19 and 21bp repeats respectively. Furthermore, IE86 is able to bind the *cis*-repression sequence (*crs*). In addition, between the unique region and the modulator exists a further promoter for the UL127 open reading frame. (Adapted from Meier & Stinski, 2006).

promoter activity (Isomura *et al.*, 2004). Activation and repression of the MIEP is regulated by the range of cellular transcription factors that bind to these described elements (see Fig. 1.8). Some factors have multiple binding sites along the enhancer, whereas other factors bind to specific repeat elements within the MIEP (17, 18, 19 and 21bp). For instance, there are at least four 18bp repeat elements in the enhancer that bind NF- κ B. Similarly, 19bp elements in the enhancer are able to bind CREB/ATF (Hunninghake *et al.*, 1989; Sambucetti *et al.*, 1989). Additionally, these 19bp elements also bind Sp1 (Lang *et al.*, 1992). The activity of each of these transcription factors is modulated by the environmental conditions set by the infected cell as well as the virus. The binding of positive factors is thought to strengthen basal transcription complex formation and aid recruitment of co-activators, such as chromatin remodelling enzymes, to the promoter. Studies of recombinant viruses with targeted mutations in the unique region or enhancer have shown little change to MIEP activity (Meier & Stinski, 1997; Pruessner & Meier, 2000; Meier, 2001). Similarly, specific mutations of the enhancer's cyclic AMP response element (CRE) or NF- κ B binding sites show minimal effects during lytic infection (Keller *et al.*, 2003; Benedict *et al.*, 2004; Gustems *et al.*, 2006). This is also true of the Sp1 binding sites, although in conjunction with the removal of Sp3 sites some effects on transcription and replication could be detected (Isomura *et al.*, 2005). However, these findings may also be due to trans-acting viral components such as pp71, UL69, TRS1/IRS1 and UL35 compensating for loss of sites at the MIEP (Liu & Stinski, 1992; Winkler & Stamminger, 1996; Romanowski *et al.*, 1997; Homer *et al.*, 1999; Bresnahan & Shenk, 2000; Schierling *et al.*, 2004). The unique region is also able to bind additional transcription factors such as PDX1 (Chao *et al.*, 2004). Similarly, the modulator element has an imperfect dyad symmetry which other factors bind specifically (Boshart *et al.*, 1985; Lubon *et al.*, 1989). These factors also have an impact on the expression of the UL127 ORF that is located in this region in the opposite direction to that of the MIEP but is not seen to be expressed during permissive infection of fibroblasts (Lundquist *et al.*, 1999; Angulo *et al.*, 2000; Lashmit *et al.*, 2004; Lee *et al.*, 2007b; Stinski & Isomura, 2008). Furthermore, the modulator has been shown to be sensitive to DNase I and this sensitivity changes depending on the differentiation status of the infected cell (Nelson *et al.*, 1987; Lubon *et al.*, 1989). Indeed, changes in binding of transcription factors may be important for differentiation-dependent regulation of permissiveness for lytic infection (Lubon *et al.*, 1989; Ghazal *et al.*, 1990; Nelson *et al.*, 1990). A number of sequence

analyses have identified putative binding sites for a range of other transcription factors that may be able to associate with the MIEP and are known to act as transcriptional repressors: modulator binding factors (Shelbourn *et al.*, 1989; Kothari *et al.*, 1991), Yin Yang 1 (YY1) (Liu *et al.*, 1994), methylated DNA-binding protein (MDBP) (Zhang *et al.*, 1991), modulator recognition factor (MRF) (Huang *et al.*, 1996), growth factor independence-1 (Gfi-1) (Zweidler-McKay *et al.*, 1996), CCAAT/enhancer binding proteins (C/EBP) (Prosch *et al.*, 2001) and Ets-2 repressor factor (ERF) (Bain *et al.*, 2003). It has been suggested that it is a balance of these activators and repressors that may be key to determining lytic or latent infection in a differentiation-dependent manner (Sinclair & Sissons, 1996). Three copies of the 21bp repeat elements in the enhancer, as well as in the upstream modulator, have been shown to mediate transcriptional repression of the MIEP in undifferentiated cells (Nelson *et al.*, 1987; Kothari *et al.*, 1991; Sinclair *et al.*, 1992; Liu *et al.*, 1994). Indeed, YY1, ERF and silencing binding protein (SBP) have been shown to bind to these repeats *in vitro* (Liu *et al.*, 1994; Huang *et al.*, 1996; Thrower *et al.*, 1996; Bain *et al.*, 2003). However, removal of the 21bp repeats, the modulator or both from the viral genome has not shown a loss of repression of MIEP activity in undifferentiated cells (Meier & Stinski, 1997; Meier, 2001), although a loss of these sites does lead to a perturbation of IE gene expression even in permissive cells (Pruessner & Meier, 2000). Hence, confirmation of the role of the 21bp repeat elements in the regulation of latency and lytic gene expression during infection still requires confirmation. Whilst mutation of CRE sites in the enhancer appears to have little effect to productive infection of a permissive cell type, stimulation of the cAMP signalling pathway by treatment with forskolin (FSK) has been shown to alleviate silencing of the MIEP in an undifferentiated non-permissive cell type (Keller *et al.*, 2007).

Although there is good knowledge of the cellular transcription factors that regulate the viral MIEP, the mechanism by which negative regulators repress MIEP activity is still unclear. However, recently, the molecular mechanisms by which the MIEP may be regulated have been investigated using the T2 and THP1 differentiation-dependent cell models. Using these cells, it has been found that the level of YY1 protein decreases during differentiation whereas, contrastingly, the steady state level of ERF does not (Pizzorno, 2001; Bain *et al.*, 2003). Thus, repression of the MIEP cannot simply be linked to the levels of repressor factors within the cells. Indeed, it is now known that it is the ability of

these factors to interact physically with and recruit chromatin remodelling enzymes that provides the ability to modulate MIEP activity (Wright *et al.*, 2005). This mechanism and process of regulation of the viral MIEP will be discussed further in section 1.10.

1.9.2 HCMV major immediate early proteins

The MIEP drives the production of the major IE primary transcript that is differentially spliced to produce five mRNA species (Shirakata *et al.*, 2002) (see Fig. 1.9). The MIE proteins produced from these transcripts include the most abundant IE72 protein (sometimes known as IE1, IE1-p72 and UL123), the IE86 protein (IE2, IE2-p86 and UL122) as well as more minor products which include p55 (IE2-p55), p38 (IE1-p38) and p18 (IE2-p18) (Wathen & Stinski, 1982; Stinski, 1993; Stenberg, 1996). Both IE72 and IE86 are conserved across the betaherpesvirus subfamily.

IE72 is composed of 491 amino acids and is encoded by exons 2, 3 and 4 of the MIE region. It can be post-translationally modified by phosphorylation and sumoylation (attachment of small ubiquitin-like modifiers (SUMO)) (Pajovic *et al.*, 1997; Xu *et al.*, 2001; Spengler *et al.*, 2002). Upon expression, IE72 localises to the nucleus of lytically infected cells and locates to ND10, causing their dispersal (see section 1.11) (Korioth *et al.*, 1996; Ahn & Hayward, 1997; Ahn *et al.*, 1998a; Wilkinson *et al.*, 1998; Muller & Dejean., 1999). IE72 is also able to interact with metaphase chromatin (Lafemina *et al.*, 1989; Ahn *et al.*, 1998a; Wilkinson *et al.*, 1998; Nevels *et al.*, 2004a; Reinhardt *et al.*, 2005). At low MOIs, IE72 deletion viruses have defects in viral early and late gene expression, viral DNA synthesis as well as genome replication and plaque formation. However, this can be compensated for by high MOIs (Mocarski *et al.*, 1996; Greaves & Mocarski, 1998; Gawn & Greaves, 2002). Much of the work carried out on IE72 has been performed using transient transfection assays and these *in vitro* analyses have suggested that IE72 is able to promote transcription from various viral and cellular promoters. The IE72 protein is thought to carry out this function in a number of ways: physical interaction between IE72 and a number of transcriptional regulators important during cell cycle (see section 1.6.5). Association of IE72 with factors involved with initiation of transcription and ND10 associated proteins also occurs (see section 1.11 below) (Hayhurst *et al.*, 1995; Margolis *et al.*, 1995; Poma *et al.*, 1996; Lukac *et al.*, 1997; Yurochko *et al.*, 1997; Ahn *et al.*, 1998a; Tang & Maul, 2003; Nevels *et al.*, 2004b). IE72 is also known to be able to

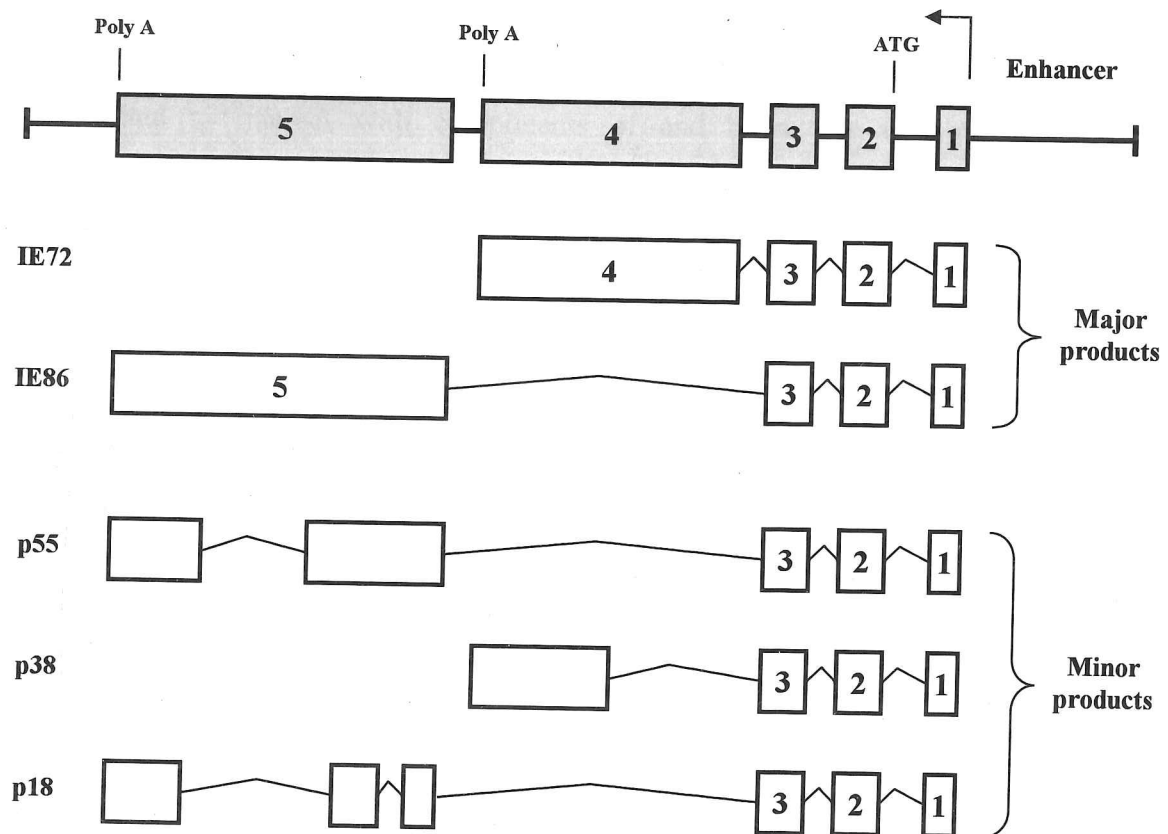


Figure 1.9 Differential splicing of the MIE region. Differential splicing of the primary transcript results in the production of a number of MIE transcripts and gene products. The products can be split into two groups: the major products (IE72 and IE86) and the minor products (p55, p38 and p18), although all share the first 3 exons (open boxes) and production of each is regulated by the enhancer. (Adapted from Shirakata *et al.*, 2002).

promote the phosphorylation of certain cellular transcription factors, including pRB, E2F family members and c-Jun via its own kinase activity. Additionally, IE72 can promote the phosphorylation of other factors through the activation of cellular kinases (JNK and cyclinE/cdk2 kinase) in addition to interaction with the RelB NF- κ B subunit (Pajovic *et al.*, 1997; Zhang *et al.*, 2003; Wang & Sonenshein, 2005). Furthermore, IE72 has also been demonstrated to interact with components of and cause the disruption of ND10, overcoming this transcriptionally repressive cellular environment that will be discussed further in section 1.11 (Muller & Dejean, 1999; Xu *et al.*, 2001; Tang & Maul, 2003; White & Spector, 2004). IE72 has also been found to direct cell cycle to an early S-like phase and impede cell apoptosis during infection, whilst it may also be able to inhibit the interferon response (Zhu *et al.*, 1995; Castillo *et al.*, 2000; Yu & Alwine, 2002). Intriguingly, IE72 is also able to stimulate transcription from the MIEP and through covalent linkage to SUMO-1 during infection via lysine 450 may also be able to increase expression of IE86 (Cherrington & Mocarski, 1989; Nevels *et al.*, 2004a). However, whilst the two MIE proteins are often found to synergistically transactivate promoters, IE72 has not been shown to physically interact with DNA or IE86 directly.

IE86 is a 579 amino acid protein corresponding to exons 2, 3 and 5 of the MIE region. IE86 is able to form homodimers and, upon lytic infection, locates to the periphery of ND10. However, it plays no role in dispersal of these domains (Macias & Stinski, 1993; Ishov *et al.*, 1997; Ahn *et al.*, 1998a). The indispensable nature of IE86 to the HCMV lytic cycle has been revealed using recombinant viruses with mutations in the protein, as with the functional homologue of MCMV, and the importance of IE86 during infection is highlighted during highjacking of the cell cycle (discussed in more detail in section 1.6.5.) (Angulo *et al.*, 2000; Marchini *et al.*, 2001). IE86 expression is vital for activation of viral early and late genes and also auto-repression during infection: due to its absolute indispensable nature all efforts to produce IE86 null mutants have failed (Marchini *et al.*, 2001; Heider *et al.*, 2002a; White *et al.*, 2004). Therefore, as with IE72, much of the information established concerning IE86 function has arisen from the use of transient expression assays. These studies have demonstrated that IE86 is able not just to act as a transactivator of a range of viral and cellular promoters but also as a trans-repressor of the MIEP itself. The gene product is able to interact with TATA boxes and specific *cis*-elements via cellular transcription factors to stimulate RNA polymerase II-dependent

transcription. These interactions occur through components of the basal transcription initiation complex, such as TBP, certain transcription factors and also histone acetyltransferases (HATs) (Hagemeier *et al.*, 1992a; Caswell *et al.*, 1993; Furnari *et al.*, 1993; Jupp *et al.*, 1993b; Lukac *et al.*, 1994; Sommer *et al.*, 1994; Spier *et al.*, 1994; Scully *et al.*, 1995; Schwartz *et al.*, 1996; Bonin & McDougall, 1997; Fortunato *et al.*, 1997; Lukac *et al.*, 1997; Bryant *et al.*, 2000). In addition, IE86 is also able to cause transcriptional activation of viral early genes, including the UL112-113 region, via binding to a 14bp sequence of DNA that is upstream to the start site of transcription (Scully *et al.*, 1995; Rodems *et al.*, 1998). Conversely, and rather intriguingly, IE86 is able to negatively autoregulate expression from the MIEP by binding the minor groove of a palindromic *cis*-repression sequence (*crs*) between -13 to -1 relative to the transcriptional start site and affect the assembly of the pre-initiation transcription complex as well as the chromatin structure of the MIEP at late times of infection (Pizzorno & Hayward, 1990; Cherrington *et al.*, 1991; Liu *et al.*, 1991; Jupp *et al.*, 1993a; Lang & Stamminger, 1993; Macias & Stinski, 1993; Wu *et al.*, 1993; Reeves *et al.*, 2006; Isomura *et al.*, 2008a). It has also been found that the ability of IE86 to act as a transcriptional activator can be modulated by post-translational phosphorylation of the protein as well as by sumoylation (Harel & Alwine, 1998; Hofmann *et al.*, 2000; Ahn *et al.*, 2001; Heider *et al.*, 2002b; Barrasa *et al.*, 2005). Interestingly, despite the importance of IE86, the protein's amino acid sequence does differ amongst HCMV strains such that transcriptional activation of target promoters can be affected in assay systems. However, it is not known whether these differences affect the life cycle of the virus (Barrasa *et al.*, 2003). Furthermore, although functions, such as inhibition of the IFN β response and post-infection chemokine expression induction, have also been attributed to the IE86 gene product (Taylor & Bresnahan, 2005; Taylor & Bresnahan, 2006) effects including inhibition of apoptosis and transformation of cells still require further work to fully determine these roles for IE86 (Lukac & Alwine, 1999).

1.10 Chromatin remodelling and regulation of gene expression

1.10.1 Introduction

It is now well recognised that the control of cellular gene expression involves not only the formation or inhibition of the transcriptional pre-initiation complex and the recruitment of certain basal transcription factors at gene promoters but also the higher order chromatin structure. As the understanding of the role chromatin structure plays in the regulation of

cellular gene expression has advanced, it has also become apparent that chromatin structure may also play an important role in the regulation of viral gene expression.

1.10.2 Chromatin, histones and the nucleosome

The term chromatin was first used by Flemming in 1882 to describe what he believed to be a nuclear scaffold that absorbed dye specifically at sites within the nucleus (Flemming, 1882). It is now well established that chromatin is a complex of double stranded DNA with histone proteins that form the sub-unit of chromatin, nucleosomes (Kornberg, 1974; Oudet *et al.*, 1975). Nucleosomes are present along a length of DNA with a characteristic 'beads on a string' appearance when viewed using an electron microscope (Oudet *et al.*, 1975) and have been found to be composed of a number of histone proteins. Specifically, the nucleosome core comprises two copies each of histones H2A, H2B, H3 and H4 arranged into an octamer that interacts directly with the DNA (see Fig. 1.10) (Chung *et al.*, 1978). A further association of histone H1 then secures the complex and its position on the length of DNA such that digestion with micrococcal nuclease first produces ~166bp particles whilst histone H1 is in place before further digestion results in ~146bp particles after the loss of protection associated with H1 (Thomas, 1999). After nucleosomal construction, the chromatin can then be further compacted into the higher order 30nm solenoid form providing the distinctive electron dense appearance of cellular chromosomes. This compaction of DNA by higher order chromatin folding permits over one metre of DNA to be accommodated within the eukaryotic nucleus. However, it also results in an accessibility dilemma for DNA replication and transcription machinery. Initially, it was proposed that chromatinised DNA was transcriptionally inactive and that removal of the nucleosome complex would be necessary for transcriptional activation (Oudet *et al.*, 1975). Later, it was found that the remodelling of chromatin structure within specific regions of DNA was playing a part in the regulation of transcription (Weintraub & Groudine, 1976; Lilley & Pardon, 1979) but that transcriptionally active DNA was not necessarily free from histone association (Kuo *et al.*, 1998). More recently, it has become established that it is biochemical modification by histone modifying enzymes of amino-terminal tails of the core histone proteins that leads to a change in the conformation of the chromatin structure (see Fig. 1.10). The possible modifications to each histone are numerous with over sixty so far detected and some will be discussed below (reviewed in: Berger, 2007; Kouzarides, 2007a) (summarised in Table 1.2). However, overall, the remodelling of the chromatin

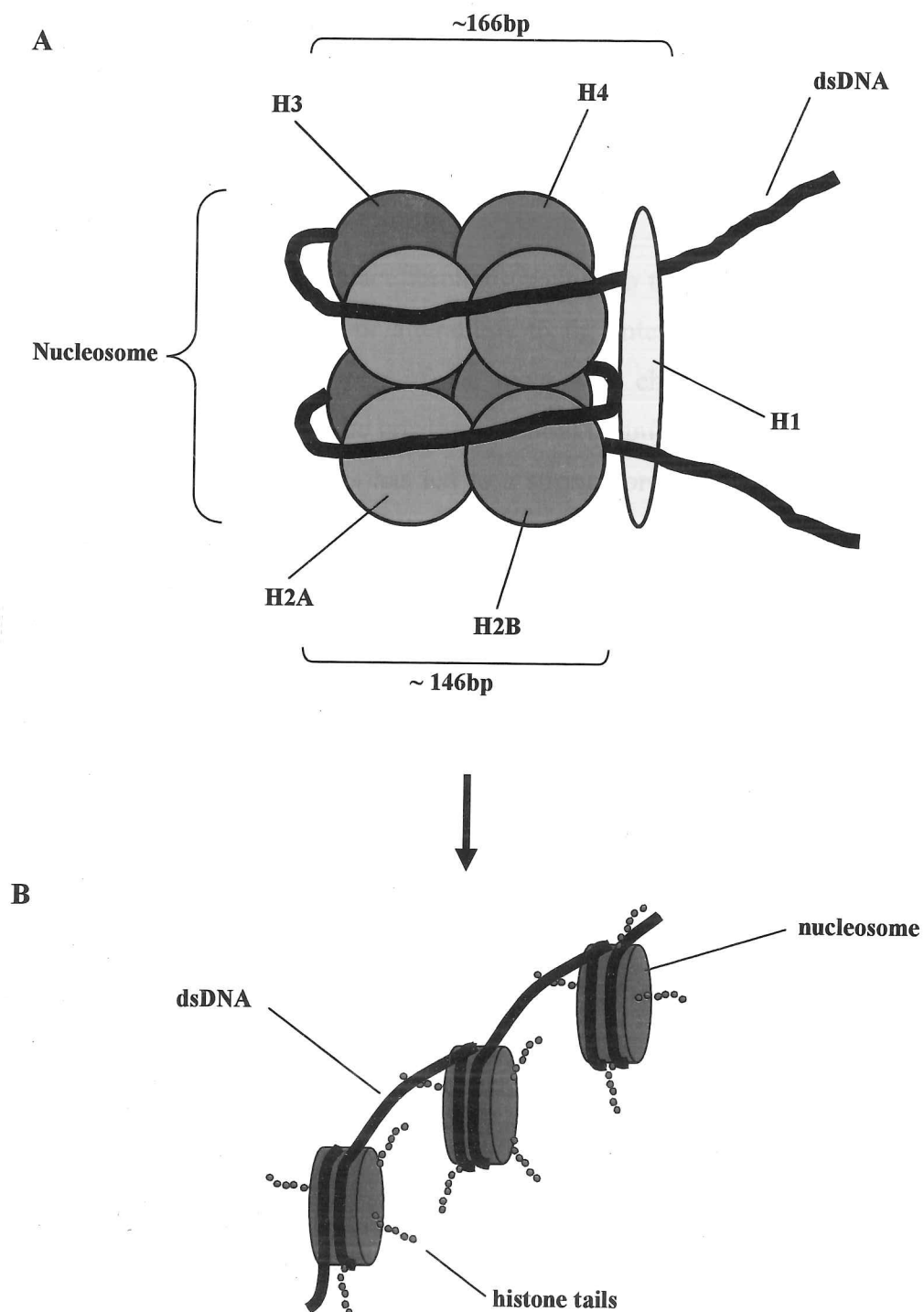


Figure 1.10 Nucleosome and chromatin structure. (A) The nucleosomal structure is composed of two copies each of histones H2A, H2B, H3 and H4 that form a complex and interact directly with double stranded DNA (dsDNA). This structure is subsequently stabilised by the association of histone H1 with the dsDNA. (B) Nucleosomes along the length of dsDNA lead to the 'beads on a string' chromatin arrangement that is visible via electron microscopy. The histone tails, shown protruding from the nucleosomal structure in the cartoon, can be modified by chromatin remodelling enzymes which lead to changes in the local chromatin structure. Nucleosome sliding along the length of the dsDNA can also occur.

structure either leads to a more open or a more closed conformation generally resulting in transcriptional activation or repression, respectively (see Fig. 1.11). This determination of the transcriptional ability of chromatin based on the quantity and quality of the histone modifications has been termed the histone code hypothesis (Strahl & Allis, 2000).

1.10.3 Histone acetylation

Chromatin remodelling can take place through changes to the contacts between different histones in adjacent nucleosomes or alterations to the interactions of histones with the associated DNA. Acetylation is one of the most well characterised post-translational modifications of histones and has the greatest potential to unfold chromatin as it neutralises the basic charge of the lysine. This has led to a strong correlation of histone acetylation with transcriptionally active regions of the eukaryotic genome (Kuo & Allis, 1998; Lusser, 2002). The acetylation of amino-terminal lysines of histone tails that are more accessible for modification takes place via the action of histone acetyltransferases (HATs). These enzymes can be divided into three main families: GNAT, MYST and CBP/p300 (Sterner & Berger, 2000). In general, the enzymes are able to modify more than one lysine, although some specificity occurs. Positively charged lysine residues are likely to form stable interactions with the negatively charged DNA producing a closed conformation of chromatin structure and repressing transcriptional activity. However, once acetylated, the association is disrupted and a more open conformation chromatin structure produced that allows access of transcription factors and the pre-initiation complex to the DNA (Cary *et al.*, 1982; Bode *et al.*, 1983; Morgan *et al.*, 1987). It has also been posited that acetylated lysine residues may also act as binding or recognition sites for bromodomains of other proteins that could allow association of chromatin remodelling enzymes, such as HATs, and components of the TF_{II}D transcription complex including TAF_{II}250 (Dhalluin *et al.*, 1999; Jacobson *et al.*, 2000; Zeng & Zhou, 2002).

As lysine acetylation, predominantly on histones H3 and H4, is associated with transcriptional activation, histone deacetylation correlates with transcriptional repression. This is likely due to a reversion in charge of histone protein and a subsequent closer association of DNA (Lusser, 2002). The process of lysine deacetylation is again carried out by a group of enzymes known as the histone deacetylases (HDACs) that can be subdivided into class I, II and IV HDACs and the class III NAD-dependent enzymes of the Sir

<i>Histone modification</i>	<i>Residue modified</i>	<i>Position of modification on histone*</i>	<i>Transcriptional function</i>
Acetylation (ac)	Lysine (K)	H3 (9, 14, 18, 56) H4 (5, 8, 13, 16) H2A H2B	Activation
Methylation (me)	Lysine (K)	H3 (4, 36, 39)	Activation
		H3 (9, 27) H4 (20)	Repression
	Arginine (R)	H3 (17, 23) H4 (3)	Activation / Repression
Deimination	Methylated Arginine (Rme)		Activation / Repression
Phosphorylation	Serine (S) Threonine (T)	H3 (3, 10, 28) H2A H2B	Activation
Ubiquitination	Lysine (K)	H2A (119) H2B (120)	Repression Activation
Sumoylation	Lysine (K)	H2A (126) H2B (6, 7)	Repression
ADP ribosylation	Glutamic acid (E)		
Proline isomerisation	Proline (P)	H3 (30-38)	Activation / Repression

* *bold modification positions highlight those used as markers for this study*

Table 1.2 Classes of histone modifications and function. A summary of the possible post-translational modifications of the amino-termini of histone tails, their position and function (Adapted from: Berger, 2007; Kouzarides, 2007)

family (Khochbin *et al.*, 2001; Gregoret *et al.*, 2004). Most examples of these enzymes, again, have little specificity for a particular acetyl group. However, some yeast enzymes have been found to target certain residues, specifically, whilst the human enzyme SirT2 has been recently demonstrated to have a preference for acetylated lysine 16 of histone H4 (H4K16ac) (Vaquero *et al.*, 2006). Although, in general histone acetylation and deacetylation is associated with transcriptional activation and repression, respectively, this is not always the case. Some promoters have been shown to become active in the absence of histone acetylation (Dudley *et al.*, 1999). Hence, certain modifications have the ability to activate or repress under different conditions and therefore it is the context of modification that can decide transcriptional fate.

1.10.4 Histone methylation

In comparison to acetylation of histones, methylation is a much more specific process that can actually occur on lysine and arginine residues in amino-terminal histone tails. Historically, methylation of histones has been associated with transcriptional silencing and has been shown to be linked with epigenetic inheritance (Razin, 1998; Lachner *et al.*, 2003). However, more recently, it has become clear that methylation can lead to both activation and repression of transcription given the context of modification (reviewed in: Bannister & Kouzarides, 2005; Klose & Zhang, 2007).

Methylation of lysine residues on histone amino-terminal tails takes place through the action of histone methyltransferases (HMTs). HMTs can append up to three methyl groups to lysine residues. This is usually catalysed by a SET domain, present in most HMTs, and the cofactor S-adenosyl-L-methionine (SAM). Lysine methylation can have both an activatory or inhibitory effect on transcription. Methylation of lysines 4, 36 or 79 on histone H3 (H3K4, H3K36, H3K79) is implicated in activation of transcription. Indeed, methylated H3K4 localises to the 5' end of active genes in yeast and is associated with transcriptional elongation with a correlation of the initiated form of RNA pol II found associated also (Schneider *et al.*, 2004). In contrast, methylation of H3K9, H3K27 and H4K20 is associated with transcriptional repression. Methylation of H3K9 has been implicated in the silencing of euchromatic genes as well as aiding the formation of silent heterochromatin and this appears to be dependent on the number of added methyl groups (Rice *et al.*, 2003). Mono- and di-methylation of H3K9 is carried out by the G9a family of

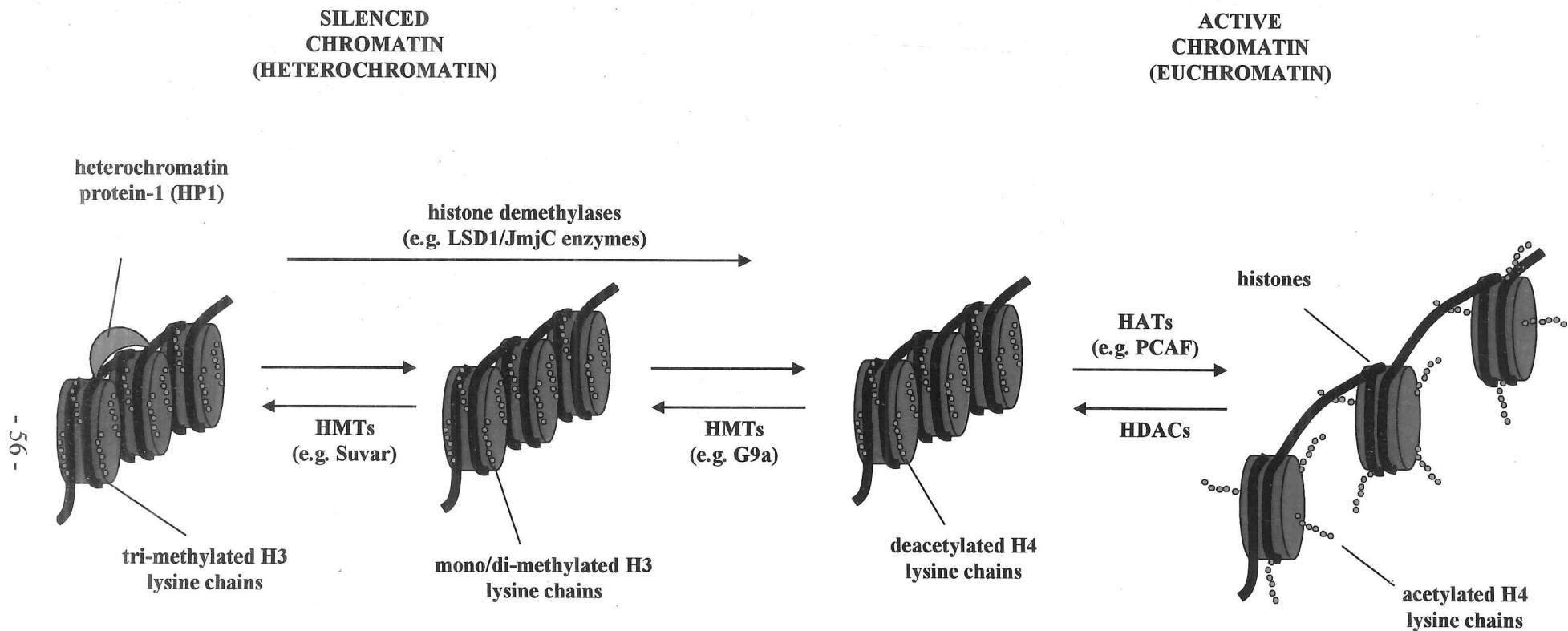


Figure 1.11 Regulation of gene expression via chromatin remodelling. Control of gene expression in eukaryotes can take place via post-translational modifications to histone proteins associated with DNA in the chromatin complex. Methylation of histone lysine chains, a marker of transcriptionally silent regions, is performed by histone methyltransferases (HMTs). Silencing is enhanced by recruitment of proteins such as heterochromatin protein 1 (HP1). Demethylation takes place via enzymes, such as LSD1/JmJc enzymes, before acetylation of histones is achieved with histone acetyltransferases (HATs). This causes a more open conformation of chromatin to arise: an environment associated with transcriptionally active regions. This open chromatin can be condensed, with deacetylation taking place via histone deacetylases (HDACs).

HMTs, whereas tri-methylation of H3K9 is performed by the Suvar family of HMTs (Rice *et al.*, 2003). The silencing of transcription via methylation at H3K9 also involves the recruitment of further markers of transcriptionally repressed chromatin, such as heterochromatin protein 1 (HP1). As the name suggest, HP1 is predominantly found at sites of condensed, inactive heterochromatin and recruitment is dependent upon methylation of the lysine residues (James *et al.*, 1989). HP1 is also found in three isoforms: HP1 α , β and γ (Jones *et al.*, 2000; Maison & Almouzni, 2004). Methylation of H3K9 or H3K27 provides a target for the chromatin binding domain of HP1. This methylation promotes binding and the avidity of binding intensifies upon increasing lysine methylation (Khorasanizadeh, 2004). Therefore, transcriptionally silent heterochromatic regions can be recognised by both the association of methylated lysine residues but also HP1 presence (Bannister *et al.*, 2001). This dogma has been recently tested with the finding that tri-methylated H3K9 (H3K9me₃) and HP1 γ are enriched in the coding region of active genes (Vakoc *et al.*, 2005). However, it is possible that this merely highlights the fact that the presence of methylated histones or chromatin associated proteins may have a different meaning when examining the coding regions to genes.

Since evidence has long existed that the action of HATs is opposed by the action of HDACs, it has been hypothesised that at least one enzyme must exist that de-methylates histone residues. The first to be discovered, lysine-specific demethylase 1 (LSD1) (Shi *et al.*, 2004), has led to a number of further such enzymes (reviewed in: Kouzarides, 2007a). As yet, the enzymes described have two types of distinct demethylase domain: the LSD1 domain and the JmjC domain. LSD1 has been shown to have both activatory and repressive effects: demethylation of H3K4 causes repression of transcription, whereas, in androgen receptor activation, demethylation of H3K9 causes transcriptional activation (Shi *et al.*, 2004; Metzger *et al.*, 2005). Activation and repression of transcription by Jumonji domain C (JmjC) enzymes has also been shown (reviewed in: Kouzarides, 2007a). Thus, it appears that the specificity of these enzymes relies upon the methylation state of lysines which they act upon but also the cofactors that they are bound to (Shi & Whetstine, 2007).

In parallel to lysine methylation, arginine methylation can also result in either transcriptional activation or repression. Arginine methylation is carried out by protein arginine methyltransferases (PRMTs), with transfer of methyl groups from the SAM

cofactor, and these enzymes are recruited to promoters by transcription factors (Lee *et al.*, 2005). Interestingly, it has been found that the presence of modification of histones by arginine methylation sometimes appears to cycle during the activation of certain genes, such as at the oestrogen-regulated pS2 promoter, although the reason for this is not fully understood (Metivier *et al.*, 2003). However, this is not the usual representation where the methylation of arginine residues on histone H4, such as H4R3, support the acetylation of the histone further and lead to transcriptional activation (Wang *et al.*, 2001). In contrast to methylated lysine residues, there is still no evidence for the presence of any proteins that are either able to specifically bind to arginine-methylated histones or reverse arginine methylation. In spite of this, other mechanisms have been shown that may antagonise arginine methylation before it is able to take place. Recently, it has been shown that arginines are able to be deiminated such that they are converted to citrulline and cannot be methylated (Cuthbert *et al.*, 2004; Wang *et al.*, 2004c). This occurs through the action of the peptidylarginine deiminase 4 (PADI4) enzyme, which has also been shown *in vivo* to deiminate mono-methylated arginines but not di-methylated residues (Wang *et al.*, 2004c). Reversing this change has not yet been described, although the cycling of residues on activating promoters, such as pS2, strengthens the argument for the existence of such enzymes (Bannister & Kouzarides, 2005).

1.10.5 Other chromatin modifications

Other modifications of histone residues and chromatin structure have been found to have effects on transcriptional regulation, although the overall functional outcome is still less well understood than those of acetylation and methylation. Phosphorylation of serine and threonine residues, mainly on histone H3, has been shown to cause activation of transcription. This may occur by promoting the acetylation of adjacent lysine residues (Lo *et al.*, 2000) or by neutralising the effects of methylation of adjacent residues (Fischle *et al.*, 2003). In addition, enzymes such as mitogen- and stress-activated protein kinase 1 (MSK1), MSK2 as well as ribosomal serine 6 kinase 2 (RSK2) have been shown to specifically target histone-H3 serine 10 (H3S10) as well as Aurora B kinase (AurKB). AurKB phosphorylation of H3S10 causes the dissociation of HP1 from tri-methylated H3K9 and as such has been implicated in transcriptional activation (Fischle *et al.*, 2005; Hirota *et al.*, 2005). However, such phosphorylation of histones also has implications for

both opening and closing of the chromatin conformation, illustrating the importance of the context of these modifications (Berger, 2007).

Ubiquitination is a very sizable modification that has only been found on histones H2A and H2B. Ubiquitination of histones has been implicated in regulatory roles during both mitosis and also transcription. The effects can be both repressive as well as activatory and may result from proteosomal degradation of ubiquitinated transcription factors (Conaway *et al.*, 2002; Zhu *et al.*, 2005; Wang *et al.*, 2004a). Ubiquitination has also been implicated in transcriptional elongation by the histone chaperone FACT (Pavri *et al.*, 2006). It is still unclear how ubiquitination specifically functions to affect transcription levels. However, it may have a direct effect on the structure of the chromatin simply due to the size of the modification, opening the conformation and leading to increased access for the basal transcription machinery. Alternatively, ubiquitination may act to recruit other proteins in a similar manner to tri-methylation of H3K9 with HP1 (Bannister *et al.*, 2001). Also, ubiquitination may also affect the outcome of further histone modifications - a hypothesis still yet to be tested fully (Zhang, 2003). In yeast models, however, enzymes have been shown necessary to antagonise specific ubiquitination of histone residues, showing that both the addition and removal of ubiquitin is essential for stimulation of transcription (Henry *et al.*, 2003). Deubiquitination of histone-H2B has also been implicated as a requirement for heterochromatic histone-H3 methylation and subsequent DNA methylation (Sridhar *et al.*, 2007), which is a known epigenetic marker associated with long term repression. Indeed, a recent finding that monoubiquitination of histone-H2B at the 5' coding region of mammalian genes supports the understanding of this marker as one of transcriptional activation (Minsky *et al.*, 2008).

Recently, the modification of histones via sumoylation has been highlighted, predominantly through studies with yeast. Initially, data suggested that sumoylation of histone-H4 *in vivo* could regulate transcription despite the presence of the putative consensus sequence for sumoylation (Nathan *et al.*, 2003; Shiio & Eisenman, 2003). Association of sumoylated histone-H4 was also found with HP1 and HDAC1 in immunoprecipitation assays and further work has identified additional modification sites on all four core histones (Nathan *et al.*, 2006). Although it is now understood that sumoylation is able to antagonise both acetylation and ubiquitination of lysine residues to

cause transcriptional repression, the role of sumoylation is still unclear. Similarly, ADP ribosylation is an ill defined histone modification with respect to transcriptional regulation. This modification can occur as mono- or poly-ADP ribosylation and is mediated by mono-ADP ribosyltransferases (MARTs) and poly-ADP ribose polymerases (PARPs) (Hassa *et al.*, 2006). Additionally, the Sir family of NAD-dependent HDACs have been shown to have a low level of ADP ribosylation activity and may represent another class of this family. The understanding of the effects of this modification on control of transcription is limited but PARP-1 has recently been shown to cause chromatin changes to the oestrogen-regulated PS2 gene during DNA repair (Ju *et al.*, 2006). More recently, it has been shown that PARP-1 and histone-H1 have similar binding specificities to nucleosomal binding and, as such, PARP-1 is able to exclude H1 from promoters to drive transcription (Krishnakumar *et al.*, 2008). Furthermore, an enzyme that causes proline isomerisation in budding yeast, FRP4, has been shown to distort the backbone of the histone-H3 polypeptide such that the levels of methylation at nearby lysine residues is affected (Nelson *et al.*, 2006), although the full consequence of this modification is, as yet, unclear.

It is clear, then, that a wide range of chromatin modifications can interact synergistically or antagonistically for the control of transcription, DNA repair, DNA replication and chromosome structure throughout the cell cycle. The majority of those described have only come to light over the last ten to fifteen years. Hence, it is likely that enzymes capable of modifying histone tails in addition to those described above will continue to be identified. It is also important to note that, at the time of writing this thesis, a new nomenclature has been proposed for chromatin-modifying enzymes. This nomenclature takes into account the targeting of other substrates, such as non-histone proteins, such that they are named more generically related to the type of enzymatic activity they perform and the type of residue they modify. In short, this has led to the following renaming: lysine demethylases – KDMs; lysine acetyltransferases – KATs; lysine methyltransferases – KMTs (fully reviewed in: Allis *et al.*, 2007; Kouzarides, 2007b). However, for the duration of this thesis, the traditional names and terms will be used.

1.10.6 Regulation of viral gene expression by chromatin remodelling

It is becoming increasingly clear that the ability of chromatin structure to regulate transcription of eukaryotic organisms also extends to the control of viral gene expression.

In work carried out over thirty years ago, it was demonstrated *in vitro* that *bona fide* nucleosome structures could be reconstituted following the incubation of phage lambda or adenovirus DNA with histone proteins (Oudet *et al.*, 1975). Subsequent work has shown that the chromatin structure of DNA viruses has a profound regulatory effect on viral transcription and this plays an important role for viruses, such as herpesviruses, in the control of gene expression during viral latency and reactivation.

Initial work suggesting that DNA viruses may be associated with histones employed electron microscopy to view the two-dimensional chromatin structure. However, more direct evidence that chromatin is associated with the control of virus gene expression has come from studies of proteins and enzymes associated with viral genomes. Probably the most extensively studied DNA virus with respect to its association with chromatin is HSV-1. Studies have revealed that chromatin structure plays an important role in the control of viral transcription both during latent and lytic infection (reviewed in: Knipe & Cliffe, 2008). Analysis of HSV-1 genomes in the brainstem tissue of a murine model first demonstrated that the latent viral genome appeared to be associated with nucleosomal chromatin (Deshmane & Fraser, 1989). In addition, viral DNA extracted from mouse dorsal root ganglia illustrated that viral genomes were not subject to significant DNA methylation but that the LAT promoter was associated with acetylated histones (Dressler *et al.*, 1987; Bloom, 2004). In contrast, histones at the HSV-1 DNA polymerase promoter were extensively methylated consistent with transcriptional repression and further analysis showing increased methylation of lytic promoters in LAT-negative compared to LAT-positive viruses (Bloom, 2004; Kubat *et al.*, 2004; Wang *et al.*, 2005). Furthermore, using a reactivation model in neuronal culture, the ICP0 promoter was shown to be sensitive to TSA treatment and, as such, likely repressed by HDACs (Arthur *et al.*, 2001). This was consistent with dorsal root ganglia explant data that demonstrates increased histone acetylation at the ICP0 promoter (Amelio *et al.*, 2006). More recently, an *in vivo* study has also shown a direct correlation between changes to LAT promoter and representative lytic gene promoter chromatin status during induced reactivation using sodium butyrate (Neumann *et al.*, 2007). Various studies have also provided evidence that chromatinisation is also important for regulation of viral gene expression during lytic infection. Analysis by both chromatin immunoprecipitation (ChIP) assay and micrococcal nuclease digestion have demonstrated that both promoters and coding regions of various viral genes appear to

be associated with chromatin and that, intriguingly, modification to histone tails can occur throughout lytic infection which is consistent with temporal gene expression of herpesvirus gene classes (Herrera & Triezenberg, 2004; Kent *et al.*, 2004; Coleman *et al.*, 2008).

Chromatin studies of herpesviruses other than HSV-1 have also been carried out. The understanding of association of the EBV genome with chromatin has been long standing (Dyson & Farrell, 1985). A number of EBV proteins appear to associate with chromatin remodelling enzymes, including EBNA-3C that interacts with HDAC1, HDAC2 and p300 (which possess HAT activity) (Radkov *et al.*, 1999; Cotter & Robertson, 2000; Knight *et al.*, 2003). Factors controlling reactivation in an EBV model system have also been identified. Using a Burkitts lymphoma (BL) cell line that has a similar pattern of gene expression to that observed *in vivo* during latent infection (Rowe *et al.*, 1987), reactivation can be induced by cross-linking surface immunoglobulins or by the addition of TGF β (Takada & Ono, 1989; di Renzo *et al.*, 1994). Reactivation of EBV was concomitant with acetylation of the latent/lytic switch protein BZLF1 and the activation of viral lytic transcription, whereas during latency the promoter appears to be deacetylated (Jenkins *et al.*, 2000). Additionally, the use of HDAC inhibitors has also shown a correlation of increased histone acetylation and expression of LMP1 in a nasopharyngeal carcinoma cell line, consistent with a repressed or poised chromatin status (Nishikawa *et al.*, 2004).

Similarly, other members of the gammaherpesviruses, such as KSHV and herpesvirus saimiri (HVS), also demonstrate chromatin modification patterns. The KSHV LANA protein has been shown to bind to the mSin3 repressor complex that has HDAC activity and the C-terminus of LANA interacts with HP1, possibly providing a mechanism whereby KSHV latency is maintained (Krithivas *et al.*, 2000; Lim *et al.*, 2003). Similar to EBV and BZLF1, the ORF50 protein Rta is responsible for the lytic cycle of KSHV (Sun *et al.*, 1998; Lukac *et al.*, 2001). Reactivation of KSHV can be induced by treatment of latently infected cells with a number of reagents such as phorbol esters or the HDAC inhibitors sodium butyrate or TSA (Miller *et al.*, 1997; Yu *et al.*, 1999; Lu *et al.*, 2003) and it was subsequently found that the ORF50 promoter is associated with a number of chromatin remodelling enzymes (Lu *et al.*, 2003). Recently, it has also been demonstrated that the closely related T-lymphotropic herpesvirus saimiri (HVS) has a similar histone modification pattern in infection of T-cells where lytic gene promoters appear repressed

(Alberter & Ensser, 2007). Thus, most examples of human herpesviruses are associated with some form of chromatin structure either during latency, lytic infection or both and this has also been examined for HCMV.

1.10.7 Regulation of HCMV gene expression by chromatin remodelling

The association of chromatin or nucleosomal-like structures with viral DNA during HCMV infection has been recognised now for more than thirty years (Kierszenbaum & Huang, 1978; St Jeor *et al.*, 1982). However, the relationship between these structures and the control of HCMV gene expression has only relatively recently been analysed. As explained in section 1.9, it is through regulation of IE gene expression from the MIEP that HCMV differentiation-dependent control of latency and reactivation appears to be mediated and there is good evidence that this regulation is mediated by higher order chromatin structure (Sinclair & Sissons, 2006; Sinclair, 2008). The differentiation-dependent control of HCMV gene expression involves the association of cellular factors known to bind to the MIEP, such as YY1 and ERF, which are able to recruit HDACs in undifferentiated cells. Upon differentiation, however, although the absolute level of these repressors is not completely reduced, the reduction in level of cofactor repressors such as HDACs does occur (Murphy *et al.*, 2002; Bain *et al.*, 2003; Wright *et al.*, 2005).

It could be predicted that the repressed state of the HCMV MIEP in undifferentiated, non-permissive cells would correlate with chromatin markers of transcriptional repression. Contrastingly, infection of differentiated, permissive cells in which IE gene expression occurs, should result in the association of the viral MIEP with transcriptionally active chromatin markers. Models of HCMV latency and reactivation have been used to test this hypothesis. As with other herpesviruses, the use of HDAC inhibitors has been employed to examine the presence of repression of HCMV in the model cell line NT2D1 (T2). Here, TSA treatment of undifferentiated cells led to the induction of IE gene expression and, interestingly, the production of progeny infectious virus after experimental infection *in vitro* (Meier, 2001). Work from our laboratory using ChIP assay analysis of the MIEP in undifferentiated T2 cells has demonstrated that the MIEP is associated with low levels of acetylated histone-H4. However, when these cells are differentiated to their permissive form, T2RA cells, the MIEP now becomes associated with high levels of acetylated histone-H4 consistent with high levels of IE gene expression within the infected cells

(Murphy *et al.*, 2002). In parallel experiments, ChIP analysis of infected, non-permissive monocytes, a site of natural carriage of the virus (Taylor-Wiedeman *et al.*, 1991; Larsson *et al.*, 1998), has provided evidence that the MIEP is associated with low levels of acetylated histone-H4 but high levels of HP1 β (Murphy *et al.*, 2002), a hallmark of transcriptional repression (Jones *et al.*, 2000). This association is then reversed upon the infection of differentiated, permissive macrophages with high levels of acetylated histone-H4 but low levels of HP1 β found associated with the MIEP (Murphy *et al.*, 2002).

Further work from our laboratory, specifically using myeloid dendritic cell (DC) progenitors or CD34⁺ cells has confirmed this correlation between MIEP transcriptional activity and chromatin structure. CD34⁺ cells are known sites of HCMV latency and experimental infection of CD34⁺ cells and subsequent ChIP analysis, as described above, has shown that the MIEP is predominantly associated with HP1 β in these cells and is transcriptionally inactive. Long term culture of these cells results in the carriage of viral genome during which time the MIEP remains associated with the repressive chromatin marker HP1 β . In contrast, differentiation of these cells to mature dendritic cells (DCs) results in the association of acetylated histone-H4 with the MIEP and the concomitant reactivation of IE gene expression (Reeves *et al.*, 2005a). Using an *ex vivo* model, further work from our laboratory has shown that cultured CD34⁺ cells from a naturally infected, healthy seropositive individual confirm these findings. The MIEP of endogenous virus in CD34⁺ cells is found associated with HP1 β but, upon differentiation to mature DCs, chromatin remodelling of the MIEP results in the association of the MIEP with acetylated H4 consistent with reactivation of IE gene expression (Reeves *et al.*, 2005b). Consequently, reactivation of HCMV from latency appears to be intrinsically linked with the differentiation status of the infected cell and the subsequent chromatin status of the MIEP.

Although it is known that differentiation-dependent regulation of HCMV gene expression is controlled, at least in part, by chromatin remodelling, it is still unclear whether chromatinisation of the MIEP is involved in regulation of this promoter during productive infection of fully permissive cell types as seen with other herpesviruses, notably HSV-1 (Herrera & Triezenberg, 2004; Kent *et al.*, 2004; Coleman *et al.*, 2008). Investigations using MCMV have provided some evidence that the chromatin state of the viral genome

may be involved in control of viral IE gene expression even in fully permissive cells. For instance, use of TSA on permissive human U373 astrocytoma cells increases both the levels of murine IE1 gene expression and the titre of the virus recovered from the cells (Tang & Maul, 2003). Consistent with this is the deposition of viral genomes upon infection at ND10 in the nucleus (Maul, 1998). These ND10 are known to recruit histone deacetylases (HDACs) and histone methyltransferases (HMTs), enzymes that could promote repression of the viral genome. Therefore, it has been suggested that activation of MCMV IE gene expression by TSA may be due to alleviation of chromatin mediated repression of the MIEP mediated by ND10. Further studies on the association of HCMV genomes with ND10 and the possible repression of IE gene expression mediated by ND10 will be further discussed below in section 1.11 and in Chapter 4.

1.11 Nuclear domain 10 (ND10) bodies

1.11.1 Introduction

Nuclear domain 10 (ND10) bodies, sometimes known as PML nuclear bodies (PML-NBs) or PML oncogenic domains (PODs), are discrete interchromosomal structures with, so far, unclearly defined cellular functions, approximately 0.2-1.0 μm in diameter (reviewed in: Bernardi & Pandolfi, 2007; Everett & Chelbi-Alix, 2007). They comprise an accumulation of several proteins, including promyelocytic leukaemia protein (PML), Sp100 and death domain associated protein (Daxx) and have been observed in most reported cell lines typically numbering between 1 to 30 per nucleus, depending on the cell type, cell cycle phase and differentiation status (Melnick & Licht, 1999; Dellaire & Bazett-Jones, 2004). Importantly, however, they are not found in the neuronal lineage (Daniel *et al.*, 1993; Flenghi *et al.*, 1995; Gambacorta *et al.*, 1996) and this is highlighted in the neuronal teratoma cell line NT2D1 (T2 cells) where ND10 are only visible after retinoic acid induced differentiation to T2RA cells (Everett, 2001; Hsu & Everett, 2001). The integrity of ND10 is dependent on PML, which recruits the other components via direct interactions (Ishov *et al.*, 1999; Salomoni & Pandolfi, 2002). Despite their interchromosomal position, the presence of nucleic acid is not necessary for their structure to be maintained as RNase and DNase treatment do not disturb their morphology or distribution (Ascoli & Maul, 1991; Stuurman *et al.*, 1992). More recently, electron microscopy studies have determined that ND10 are actually composed of a ring-like structure that does not contain nucleic acid within the ring, although extensive contact is made with chromatin fibres through the

periphery of the ring, which may aid the maintenance of structural integrity (Boisvert *et al.*, 2000; Dellaire & Bazett-Jones, 2004; Eskiw *et al.*, 2004). These contacts appear not to be random with the domains becoming established in genomic regions that are rich in genes that are transcriptionally active (Wang *et al.*, 2004b). Although the domains appear to be reasonably uniform, ND10 are structurally and functionally heterogeneous and are dynamic structures (Bernardi & Pandolfi, 2007).

ND10 were first recognised and investigated due to their association with disease (Bernstein *et al.*, 1984). However, primarily, ND10 are linked with a form of myelogenous leukaemia known as acute promyelocytic leukaemia (APL) from where PML itself gained its name. APL is characterised by a block in myeloid cell development at the promyelocyte stage where, in 98% of cases, PML is fused to the alpha-retinoic acid receptor (RAR α) as a result of chromosomal translocation (Melnick & Licht, 1999). The ND10 then become disrupted resulting in a microparticulate pattern in the nucleus and cytoplasm (Kastner *et al.*, 1992; Dyck *et al.*, 1994; Weis *et al.*, 1994). However, despite the association of disease and the great amount of investigation focussed on these domains providing insight into the correlation with regulation of various cellular functions such as the induction of apoptosis, cellular senescence, inhibition of proliferation, maintenance of genome stability, nuclear waste depots, sites of transcriptional regulation and DNA replication and antiviral responses (Ching *et al.*, 2005; Bernardi & Pandolfi, 2007), the molecular biology of both the ND10 and its constituents remain poorly understood.

1.11.2 ND10 dynamics

ND10 not only have dynamic structures but also undergo significant changes in number, size and position under both normal circumstances and in response to cellular stress. Most ND10 appear relatively static during interphase of the cell cycle, most likely due to interactions with surrounding chromatin. Although, one study has shown that a group of smaller bodies, constituting 12% of the total ND10, show rapid ATP-dependent movement (Muratani *et al.*, 2002). However, this study used GFP tagged Sp100 (speckled protein of 100kDa - discussed further in section 1.11.3) as a marker rather than the more definitive PML and others have shown that some small foci containing Sp100, but specifically not PML, are highly dynamic (Wiesmeijer *et al.*, 2002). Hence this high dynamism may be specific to nuclear bodies only rich in Sp100. One hypothesis for the abundance of these

highly motile structures is that they function to detect foreign proteins and then promote their degradation via the proteasome, which some of the bodies have been found to interact with (Anton *et al.*, 1999; Lallemand-Breitenbach *et al.*, 2001; Dino Rockel & von Mikecz, 2002). However, this hypothesis requires additional analysis. Further experimentation has also shown that although ND10 are predominantly static, PML itself is a dynamic component as it can exchange between bodies or the nucleoplasm (Wiesmeijer *et al.*, 2002).

ND10 can not only undergo significant changes during both cell cycle progression but also in response to DNA damage and cellular stress. The number of visible bodies during G₀ phase of the cell cycle is low, but then this number increases as the cell enters G₁ phase and proceeds to S and G₂ phase (Chang *et al.*, 1995; Koken *et al.*, 1995; Terris *et al.*, 1995; Kurki *et al.*, 2003). The change in quantity of ND10 appears to be due to modifications of the surrounding chromatin environment that pull the ND10 structure apart during chromosome retraction during early S phase. Consequently, there are almost twice as many ND10 during G₂ phase as G₁ phase (Dellaire *et al.*, 2006b). It is thought that this change in appearance may have a surveillance function during replication of DNA, which is likely to be linked with their function to DNA damage (see below). A further change in ND10 morphology, specifically during mitosis, is that their ability to form large aggregates, although the PML becomes de-sumoylated and other components, such as Sp100 and hDaxx, subsequently do not associate with them (Dellaire *et al.*, 2006c). This major change to the ND10 body, and possibly its function, has led to the reclassification of this PML-based body as a mitotic accumulation of PML protein (MAPP) during mitosis. As mentioned above, ND10 are also able to respond to a number of different stimuli including DNA damage and cellular stress. Briefly, ND10 are able to redistribute into numerous smaller punctuate dots in response to heat shock, heavy metals and DNA damaging agents such as ultraviolet (UV) light, cisplatin and alkylating agents (Maul *et al.*, 1995; Eskiwi *et al.*, 2003; Nefkens *et al.*, 2003; Seker *et al.*, 2003; Conlan *et al.*, 2004; Salomoni *et al.*, 2005). However, other DNA damaging agents such as γ -irradiation lead to an increase in the number of normal size domains whilst doxorubicin (an anthracycline antibiotic that can intercalate DNA) causes an accumulation of PML around the nucleolus (Carbone *et al.*, 2002; Bernardi *et al.*, 2004; Dellaire *et al.*, 2006a). Again, although it is not yet clear why

such changes occur, ND10 appear to sense cellular stress and it is likely that they function to act against specific stresses to prolong the life of the cell.

1.11.3 ND10 constituent proteins

ND10 appear to have multiple functions and this most likely depends upon the constituent proteins at any one time. Over fifty proteins have been reported to interact with the PML protein apparently identifying them as a component of ND10. However, a number of these studies have used transient transfection and over-expression of the protein of interest and it is known that ND10 act as a sink for over-expressed proteins (Melnick & Licht, 1999; Maul *et al.*, 2000; Tsukamoto *et al.*, 2000; Borden, 2002). Hence, the importance of the association of such proteins with ND10 may be erroneous. Perhaps more meaningful data has been provided by the use of antibodies to detect endogenous levels of constituents and using cell lines stably transfected with potential ND10 constituent proteins. These types of studies have led to a much smaller number of constituents known to be present at ND10 at endogenous levels of expression: PML, Sp100, Daxx, BLM, SUMO and NDP55 (Negorev & Maul, 2001).

As already stated, the major and defining constituent of ND10 is PML. PML is part of the tripartite motif (TRIM) family of proteins defined by a zinc-finger called the RING motif, two additional B-boxes or zinc-finger motifs and a coiled coil domain, such that they are also known as RBCC family proteins (Borden *et al.*, 1995; Jensen *et al.*, 2001). The PML (or TRIM19) gene contains nine exons and alternative splicing leads to the generation of several isoforms which have been classified into seven groups (PML I-VII). All isoforms share the N-terminal region of the protein, including the TRIM, but have differing C-terminal regions and, as such, some lack the nuclear localisation signal (NLS) and are found predominantly in the cytoplasm (Jensen *et al.*, 2001; Reymond *et al.*, 2001). Rather like the numerous ND10 or ND10-like bodies found within the nucleus, the number of PML isoforms may reflect the number of possible functions and localisation of isoforms (Beech *et al.*, 2005; Condemine *et al.*, 2006). PML-IV is highly multifunctional and has been found to induce premature cell senescence through binding of p53, promote Myc destabilisation and cellular differentiation as well as binding to PU.1 and inducing PU.1-mediated transcription (Fogal *et al.*, 2000; Bischof *et al.*, 2002; Buschbeck *et al.*, 2007; Yoshida *et al.*, 2007). Other isoforms appear to have a more moderate number of roles:

PML-I is known to interact with the transcription factor AML1 to enhance AML1-induced transcription (Nguyen *et al.*, 2005); PML-III interacts with the centrosome (Xu *et al.*, 2005); and a cytoplasmic isoform of PML has also been implicated in the regulation of TGF β signalling (Lin *et al.*, 2004b).

The formation of ND10 is dependent not only on the presence of PML but also post-translational modification of PML such as sumoylation. PML is able to directly bind to the small ubiquitin-like modifier (SUMO) and the SUMO-conjugating enzyme UBC9 that causes sumoylation of PML on three lysine residues (K65, K160 & K490) and has been shown to be necessary for formation of ND10; UBC9 knockout cells have defective or absent ND10 (Borden, 2002; Nacerddine *et al.*, 2005). Whereas ubiquitin or ubiquitin-like tags usually target proteins for degradation, sumoylation appears to target proteins to ND10 (Melchior, 2000). UBC9 interacts with the RING domain of PML and covalently attaches to at least the SUMO-1 protein and likely the SUMO-2 and -3 proteins that are also able to modify PML and form polymeric structures that have been found to be able to regulate the nuclear accumulation and structure of ND10 (Kamitani *et al.*, 1998; Duprez *et al.*, 1999; Ishov *et al.*, 1999; Ayaydin & Dasso, 2004; Fu *et al.*, 2005; Shen *et al.*, 2006; Mukhopadhyay *et al.*, 2006). Upon sumoylation, PML can interact with other ND10 constituents, such as Sp100 and Daxx, through the SUMO-binding domain and their recruitment to ND10 takes place (Ishov *et al.*, 1999; Shen *et al.*, 2006).

There appears to be a profound regulation of ND10 structure during cell cycle such that ND10 are disassembled during mitosis through de-sumoylation of PML (Sternsdorf *et al.*, 1997; Dellaire *et al.*, 2006c). Then, during interphase of the cell cycle, PML becomes sumoylated and is able to interact with other PML molecules via the SUMO-binding motif, initiating the formation of new ND10 (Shen *et al.*, 2006; Heun, 2007). PML is also able at this stage to interact with other ND10 constituent proteins and recruit them to the structure, an example being Daxx that is targeted to ND10 via interaction with PML through its SUMO-binding domain whilst also being modified by SUMO (Lin *et al.*, 2006).

A permanent constituent protein of ND10, discovered through investigation using sera from patients suffering from primary biliary cirrhosis, is Sp100 (Szostecki *et al.*, 1990). Although Sp100 is able to be sumoylated, SUMO modification does not appear to be

essential for localisation of the protein to ND10 (Sternsdorf *et al.*, 1999). Like PML, Sp100 is expressed in the absence of IFN but its expression is increased by IFN treatment (Guldner *et al.*, 1992; Chelbi-Alix *et al.*, 1995). Sp100 is also similar to PML in that alternative splicing of the primary transcript gives rise to several isoforms of Sp100, some of which are able to bind DNA (Sp100-B & Sp100-HMG) (Guldner *et al.*, 1999; Bottomley *et al.*, 2001). Indeed, Sp100-B has been shown to act as a transcriptional repressor and, in addition, the Sp100-A isoform is able to interact with HP1 isoforms α , β and γ highlighting the possibility that Sp100 may play a role in the control of gene expression of locally associated DNA or foreign DNA targeted to ND10, such as virus genomes (Seeler *et al.*, 1998; Isaac *et al.*, 2006; Wilcox *et al.*, 2005; Negorev *et al.*, 2006). Intriguingly, a number of other ND10 components are also associated with the regulation of chromatin structure and control of gene expression. The chromatin modifier and histone chaperone HIRA has been found associated with ND10 as well as a SWI/SNF chromatin remodelling family member ATRX and Bloom's Syndrome DNA helicase (BLM) mentioned above (Negorev & Maul, 2001; Ishov *et al.*, 2004; Zhang *et al.*, 2005; Luciani *et al.*, 2006). Furthermore, and interestingly, is the association of the Death domain-associated protein or Daxx as it is more commonly known. Daxx acts as a multifunctional protein and was first identified due to its association with the control of apoptosis (Yang *et al.*, 1997). However, with more than ten years of research into this association, the published literature remains contentious. Daxx has more recently become prominent due to its function as a transcriptional repressor, possibly through interactions with chromatin remodelling enzymes such as HDACs (Hollenbach *et al.*, 2002). Due to the focus of this thesis, Daxx will be discussed in detail below and in Chapters 4 and 5.

1.11.4 RNA virus-ND10 interactions

Since the regulation of components of ND10 are controlled, at least in part, by IFN levels, it is not surprising that ND10 have been suggested to play a role in the control of virus infections (reviewed in: Everett & Chelbi-Alix, 2007). RNA viruses have been found not just to be affected by the components of ND10 but also to combat their effect. The methods by which PML is able to confer resistance to certain RNA virus infections falls into two groups: p53-independent and p53-dependent. IFN treatment of cells or exogenous expression of PML III isoform in p53 inactive cells lines (CHO & U373MG) leads to a decrease in the level of the retrovirus human foamy virus (HFV) gene expression through

an interaction of the N-terminal region of the viral transactivator, Tas, and subsequent suppression of viral DNA binding ability (Regad *et al.*, 2001). Similar studies using over-expression of PML III isoform have also shown the sensitivity of vesicular stomatitis virus (VSV) and influenza virus to this ND10 defining constituent. PML III appears to inhibit the replication of both viruses with effects on both mRNA and protein expression most likely through interaction with other viral or cellular proteins that regulate viral replication (Chelbi-Alix *et al.*, 1998). Interestingly, though, a varied response of influenza virus to PML isoforms has been found: over-expression of PML IV and VI in Caco-2 cells also causes resistance to virus infection, whereas siRNA treatment to down-regulate all the PML isoforms causes an enhancement of viral replication (Iki *et al.*, 2005). However, results of this type appear to be particularly cell specific since replication of influenza virus in wild type and PML^{-/-} MEFs is equally effective (Engelhardt *et al.*, 2004). Conversely, PML^{-/-} MEFs are more sensitive than wild type MEFs to rabies virus infection, although exogenous expression of PML III has little effect on viral replication (Blondel *et al.*, 2002). This was also found to be true upon analysis of lymphocytic choriomeningitis virus (LCMV) infection, whereas the ability of IFN to confer resistance to LCMV infection of PML^{-/-} MEFs compared to wild type cells was higher (Djavani *et al.*, 2001; Asper *et al.*, 2004). Indeed, although the precise PML isoforms at work are unknown, PML deficient mice also show greater susceptibility to LCMV and VSV infection (Bonilla *et al.*, 2002).

PML is also able to convey resistance to RNA virus infections through p53-dependent mechanisms. Infection of p53^{-/-} mice with VSV results in higher virus loads and morbidity than in wild type mice most likely through the untimely induction of apoptosis (Takaoka *et al.*, 2003). In addition, infection of cells with poliovirus has also been shown to involve p53: poliovirus infection induces activation of Mdm2 and Noxa in a PML-dependent manner, ultimately causing apoptosis and thus inhibiting virus replication. Inhibition of virus infection is also enhanced by over-expression of PML III. This is abolished when siRNA technology is used to down-regulate the expression of p53 or PML (Pampin *et al.*, 2006). Poliovirus is, however, able to act against this anti-viral ND10-mediated response. Specifically, poliovirus infection induces the phosphorylation of PML and also increases sumoylation of PML. This results in an increased PML-dependent recruitment of p53 to ND10, in addition to Mdm2 and proteasome components, such that degradation of p53 occurs (Pampin *et al.*, 2006). Other viruses also have mechanisms of action against the

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anti-viral defence of ND10 constituents. Although rabies virus replicates in the cytoplasm of the cell, it is able to cause reorganisation of ND10 into larger and denser aggregates (Blondel *et al.*, 2002). This occurs through a direct interaction of the viral phosphoprotein P, and its amino-terminal truncated product P3, with PML. This sequestration of PML by P results in co-localised foci in the cytoplasm with P3 causing the increase in ND10 size (Blondel *et al.*, 2002). Furthermore, the 11kDa RING finger protein, Z, of LCMV is able to interact with PML and cause delocalisation from ND10 to the cytoplasm thereby counteracting the anti-viral environment (Everett & Chelbi-Alix, 2007). Additionally, work with core protein from hepatitis C virus (HCV) has shown that it co-localises with PML and p53 in ND10 and thus may counteract the pro-apoptotic function of p53 here (Herzer *et al.*, 2005). However, studies utilising HCV infected cells have subsequently found no association of viral core protein with ND10, although an induction of certain ND10 constituents does appear to occur through the presence of core protein. Thus, further investigation is required to establish a function, if any, of this interaction (Nguyen *et al.*, 2006; Rouille *et al.*, 2006). Evidence that HIV is able to redistribute components of ND10 upon infection has also been reported (Turelli *et al.*, 2001). However, one investigation of the accumulation and intra-nuclear distribution of unintegrated HIV-1 DNA showed that the virus doesn't modify ND10 at either early or late times of infection (Bell *et al.*, 2001). Although more recent data does support an interaction of the retroviral integrase protein with Daxx that will be discussed further in Chapter 4 (Greger *et al.*, 2006).

1.11.5 DNA virus-ND10 interactions

The interaction of viruses with ND10 was first highlighted by the observation that HSV-1 was able to disrupt the integrity of ND10 through the viral immediate early protein ICP0 (Maul *et al.*, 1993) and later through the finding that the HSV-1, HCMV and adenovirus genomes become associated with ND10 after infection (Ishov & Maul, 1996; Maul *et al.*, 1996). Since then, a number of other members of DNA virus families have been shown to have gene products that interact with and modulate the components and structure of ND10 (reviewed in: Everett, 2006; Everett & Chelbi-Alix, 2007). The interaction of herpesviruses and ND10 will be discussed separately below (see section 1.11.6), whilst other DNA viruses interactions will be focussed upon in this section.

Upon infection of cells with adenoviruses, the viral proteins E1A and E4orf3 are able to traffic to ND10. The ND10 in these cells are subsequently disrupted and early work showed that E4orf3 was sufficient for this disruption with later work providing evidence that the PML II isoform mediates this effect (Carvalho *et al.*, 1995; Doucas *et al.*, 1996; Ishov & Maul, 1996; Hoppe *et al.*, 2006). This PML/E4orf3 interaction reorganises ND10 into thread-like structures, further including the E1B-55 protein. This has been suggested to prevent the restrictive effects of the cellular DNA repair machinery rather than particularly augment viral gene expression (Doucas *et al.*, 1996; Stracker *et al.*, 2005). Another study has suggested that E1B-55 could be a target for sumoylation by SUMO-1 and subsequently be targeted to ND10 and that E4orf6 was then able to recruit E1B-55 away from ND10 (Lethbridge *et al.*, 2003). This corresponds well with the known function of E1B-55, whereby it is able to interact with both the ND10 component protein Daxx and p53 and remove them from ND10 (Zhao & Liao, 2003; Zhao *et al.*, 2003). This has led to the hypothesis that viral proteins help transformation of the infected cells, although the inhibition of apoptosis by sequestering the two cellular factors Daxx and p53 seems more likely. Nevertheless, the E4orf6 and E1B-55 proteins have also shown multifunctional properties, whereby their presence during infection is able to play some role in the control of mRNA trafficking and certainly inhibition of cellular mRNA nuclear export (Flint & Gonzalez, 2003).

Another group of large DNA viruses known to interact with ND10 after infection are the papillomaviruses, although the importance of these interactions for virus infection is still not fully understood. It was first observed that the minor capsid protein, L2, of bovine papillomavirus (BPV) associated with ND10 and that L2 was able to recruit the major capsid protein, L1, and the regulatory protein, E2, in transfected cells (Day *et al.*, 1998). Work with human papillomavirus (HPV) subsequently provided evidence that ND10 modified to act as a site of replication for the virus with regulatory proteins E1 and E2 found along with replicating viral DNA at ND10 (Swindle *et al.*, 1999). The L2 and E4 virus proteins have also both been implicated in reorganisation of ND10 into a replication compartment: E4 via interaction with PML; and L2 through recruitment of Daxx but removal of Sp100, possibly with degradation of the protein (Florin *et al.*, 2002; Roberts *et al.*, 2003). Although recent work has shown in biologically relevant raft cultures that HPV proteins and viral DNA are associated with a higher number of ND10 visible in upper

differentiated cells than normal uninfected cells (Nakahara & Lambert, 2007), further investigation is necessary to fully establish the importance of ND10 as a site of HPV replication.

The replication of papovaviruses, such as simian virus 40 (SV40), has also been shown to involve ND10. The large T antigen of SV40 activates viral DNA replication through binding to the viral origin of replication, whilst localising in foci juxtaposed to ND10 (Carvalho *et al.*, 1995; Doucas *et al.*, 1996; Jiang *et al.*, 1996). Indeed, viral transcription has been shown to occur near ND10, although this may be a consequence of the genome being targeted to ND10 for viral DNA replication (Tang *et al.*, 2000). Interestingly, using aphidicolin treatment to inhibit viral DNA replication or molecular disruption of the SV40 origin of replication is concomitant with the absence of ND10 association of the viral genome, underpinning the necessity for the interaction of large T antigen with the viral genome for this association (Tang *et al.*, 2000).

Other viruses that require the presence of a helper virus for full replication have also been found to have some association with ND10 during replication. Adeno-associated virus (AAV), a parvovirus, which requires another virus, such as HSV-1 or adenovirus, for replication also shows an interaction with ND10. However, discriminating between the mechanisms of replication for two viruses within one infected cell can prove problematic. Assays that allowed visualisation of AAV replication in live cells have allowed determination that AAV replication compartments can associate with modified ND10 in adenovirus infected cells. Conversely, in HSV-1 co-infected cells, association of replication compartments could not be found with ND10, most likely due to the complete disruption of ND10 caused by ICP0 (discussed further in section 1.11.6). Importantly, the use of an ICP0-null mutant of HSV-1 still resulted in the formation of AAV replication compartments. Consequently, the disruption of ND10 appears not to be a prerequisite for AAV replication (Fraefel *et al.*, 2004). Further, it has been shown that only certain elements of the herpesvirus replication machinery are sufficient for replication of a plasmid carrying the AAV-2 genome: the helicase primase complex (UL5, UL8 & UL52) and the single-stranded DNA binding protein (UL29) (Weindler *et al.*, 1991). However, in the absence of a lytically replicating helper virus, AAV integrates into the host genome and becomes latent. Another study also found that the localisation of ICP0 to ND10 and their

disruption was not required for the activation of the AAV replication promoter, whereas binding of ICP0 to the herpesvirus-associated ubiquitin-specific protease (HAUSP) made a significant contribution to reactivation from the latent state of AAV (Geoffroy *et al.*, 2004). Once again, though, it could be theorised that this interaction is important in modification of p53 levels and subsequent effects on the infected cell since it is known that HAUSP is able to cause accumulation and modification of p53 (Li *et al.*, 2002).

1.11.6 Herpesvirus-ND10 interactions

Since the first discovery that HSV-1 genomes locate near to or at ND10 during infection and that ICP0 is necessary and sufficient to disrupt the ND10 structure (Maul *et al.*, 1993; Ishov & Maul, 1996; Maul *et al.*, 1996), members of all the *herpesviridae* have been examined for their ability to alter ND10 during infection (reviewed in: Everett, 2006; Everett & Chelbi-Alix, 2007). It has been found that all herpesvirus subfamilies do, indeed, interact with ND10, although in different manners and through different mechanisms.

HSV-1 has been studied most extensively regarding its association with ND10. As alluded to above, HSV-1 genomes not only locate at ND10 but also encode gene products sufficient to disrupt the ND10 structure itself. ND10 components become associated with HSV-1 genomes as they enter the cell resulting in the formation of ND10-like bodies on the viral genome (Everett *et al.*, 2004; Everett & Murray, 2005). Neither viral transcription nor *de novo* protein synthesis is required for establishment of these ND10-like structures (Everett *et al.*, 2007). This accumulation of cellular protein at the viral DNA is thought to be an intrinsic antiviral cellular response but is a transitory one due to the synthesis of ICP0 and disruption of ND10. It has been determined that two domains of ICP0 are important for this function of ND10 disruption: near the C-terminal end of the protein is a region responsible for targeting ICP0 to ND10 after synthesis, whilst a RING-finger domain that holds ubiquitin E3 ligase activity is sufficient for inducing the degradation of PML and SUMO-modified isoforms of Sp100 (Everett & Maul, 1994; Maul & Everett, 1994; Boutell *et al.*, 2002). The importance of ND10 disruption by HSV-1 for efficient infection is reinforced by the observation that infection of cells with an ICP0-null mutant, which cannot disrupt ND10, is severely impeded but that knockdown of PML and/or Sp100 allows efficient infection with the mutant virus (Everett *et al.*, 2006; Everett *et al.*, 2008). However, cell-specific differences in ICP0-mediated PML degradation exist which

appear to depend on the particular isoform of PML examined. In Hep2 cells, the SUMO-1 modified isoforms of PML are preferentially destabilised compared to the unmodified isoforms, whilst in human foetal lung (HFL) cells all isoforms of PML are readily degraded (Everett *et al.*, 1998a; Parkinson & Everett, 2000). Additionally, although degradation of PML via ICP0 interaction involves the proteasome, one study has reported that in *in vitro* assays ICP0 alone does not ubiquitinate PML or interact with it directly, such that *in vivo* degradation of PML via ICP0 is likely to occur indirectly, possibly requiring other factors (Boutell *et al.*, 2003). Nevertheless, despite these discrepancies, the disruption of ND10 by HSV-1 ultimately leads to an environment conducive to full lytic infection (Everett, 2000). Indeed, other members of the alphaherpesviruses, such as VZV, pseudorabies virus (PRV) and type 1 bovine and equine herpesviruses, have also been found to encode orthologues of ICP0 that disrupt ND10, with their function dependent on a RING finger domain (Parkinson & Everett, 2000).

In contrast to the prototypic alphaherpesvirus HSV-1, the genome of the prototypic gammaherpesvirus EBV does not become associated with ND10 until lytic infection occurs (Sugden & Warren, 1989). Upon lytic activation ND10 become disrupted in a sequential manner with Daxx, Sp100 and NDP55 with a slower dispersal of PML before viral replication compartments develop in association with the ND10 remnants (Bell *et al.*, 2000; Amon *et al.*, 2006). Other work has pinpointed that the expression of the immediate early protein BZLF1 and subsequent SUMO-1 modification is sufficient for PML dispersal and the dispersal of Sp100 staining from ND10 (Adamson & Kenney, 2001; Deng *et al.*, 2001). More recent work has established that EBNA-LP is also able to disperse Sp100 and HP1 α from ND10 and it was hypothesised that this may function to make Sp100-A isoform available to aid EBNA2 mediated stimulation of viral gene expression (Ling *et al.*, 2005). Other gammaherpesviruses have also been shown to interact with ND10 and form replication complexes, with this best typified by KSHV (HHV-8). Whilst the genome is found associated with cellular DNA through latency, during lytic infection the K8 early protein of KSHV has been shown to target and interact with PML at ND10 although ND10 structure is not necessarily affected. (Ballestas *et al.*, 1999; Cotter *et al.*, 1999; Katano *et al.*, 2001; Wu *et al.*, 2001a).

Parallel studies have also been performed on betaherpesviruses. Analysis of the HHV-6 subtypes have found that their IE1 protein traffics to and forms a stable association with ND10 (Stanton *et al.*, 2002; Gravel *et al.*, 2004). However, this does not result in ND10 disruption. In contrast, infection with HHV-6B results in a decrease in the number of ND10, although PML levels increase and the viral transcriptional activator U19 localises to the centre of the enlarged ND10 structures (Kofod-Olsen *et al.*, 2008). Whilst the full importance of ND10 for lytic infection of the HHV-6 subtypes has not been determined, the significance of ND10 association and disruption is much more fully understood in the prototypic herpesvirus infection with HCMV.

Analagous to HSV-1, the genome of HCMV has been found to reside near to or at ND10 during lytic infection (Ishov & Maul, 1996; Maul *et al.*, 1996; Maul & Negorev, 2008). Although a precise position of the viral genome during latency has not yet been confirmed, it is also likely to reside near to ND10. Investigation of HCMV has also shown that the immediate early protein IE72 is necessary and sufficient for disruption of ND10 during lytic infection and that this is mediated by the C-terminal acidic region of IE72 which interacts with the N-terminal RING finger domain of PML to inhibit oligomerisation of ND10 components and cause PML dispersal (Kelly *et al.*, 1995; Koriath *et al.*, 1996; Ahn & Hayward, 1997; Ahn *et al.*, 1998; Wilkinson *et al.*, 1998; Lee *et al.*, 2007a). However, unlike ICP0, IE72 mediated disruption of ND10 does not result from targeting of PML for proteosomal degradation. Instead, PML appears simply to be relocalised with little evidence of change to the biochemical structure of the protein, although subsequent desumoylation of PML may be promoted after dispersal to inhibit regeneration of ND10 (Ahn *et al.*, 1998a; Muller & Dejean, 1999; Kang *et al.*, 2006). Furthermore, work using permissive U373 cells stably transfected with PML demonstrated that higher levels of PML lead to a delay of up to 6 hours for ND10 disruption and DNA replication compartment formation (Ahn & Hayward, 2000). These observations, together with observations of HSV-1, have implicated ND10 as sites of intrinsic intracellular antiviral environments. Consistent with this, targeting of ND10 by HCMV factors appears to be necessary for efficient productive HCMV infection to occur. Studies have shown that the UL82 encoded HCMV tegument phosphoprotein pp71 traffics and accumulates at ND10 via interaction with human Daxx (hDaxx) (Hofmann *et al.*, 2002; Ishov *et al.*, 2002; Schierling *et al.*, 2004). pp71 then acts as a transactivator and is able to regulate and

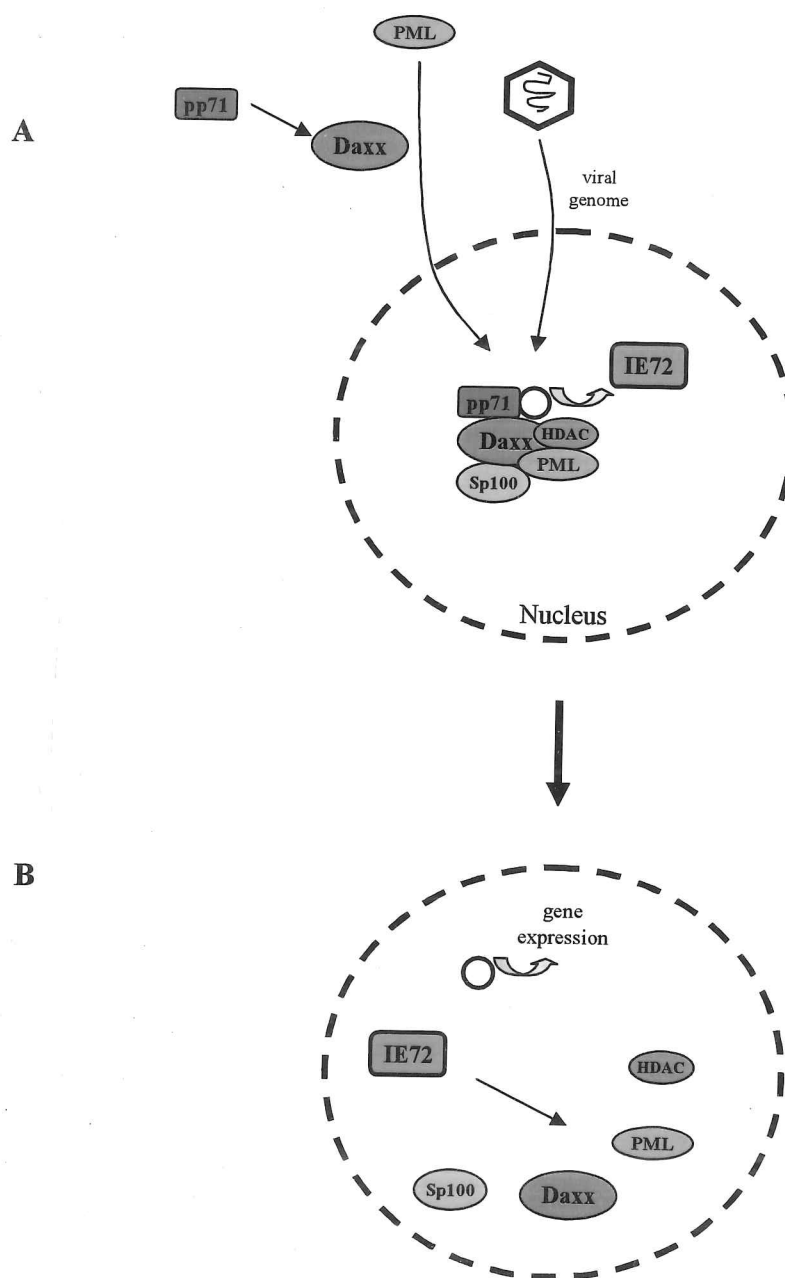


Figure 1.12 Model of HCMV interactions with ND10. (A) On entry of the virus to the cell, tegument proteins are released and the capsid traffics to the nucleus before releasing the viral genome which circularises and becomes located near to or at ND10, sites of known chromatin remodelling enzymes and transcriptional repressors. pp71 is able to interact with Daxx, which itself is able to interact with PML and transit to ND10. At ND10, pp71 is able to transactivate the MIEP of the viral genome and IE72 is expressed. (B) Expression of IE72 and targeting to ND10 leads to the PML-dependent disruption of ND10, release of other transcriptional repressors and subsequent continuation of viral gene expression.

activate viral IE transcription, before high level expression of IE72 disrupts ND10 (see Fig. 1.12) (Marshall *et al.*, 2002). Previous work from our laboratory has also shown that permissive U373 cells stably transfected with hDaxx were completely refractory to viral gene expression during infection (Woodhall *et al.*, 2006). Hence, hDaxx appears to have a profound role during infection with HCMV.

1.12 Project remit

Investigations of HCMV latency and reactivation suggest that transcriptional regulation of IE gene expression is mediated by chromatin remodelling. Additionally, investigation into lytic infection with MCMV has also suggested the importance of chromatin remodelling of the viral genome. This regulation may be mediated through ND10, a site where the viral genome is deposited after virus entry, and might involve an initial repressive cellular environment that needs to be overcome for full initiation of viral gene expression. Therefore, this thesis will detail analyses which investigate the chromatinisation of the viral MIEP during productive infection and whether chromatin remodelling is involved in regulation of viral IE gene expression during lytic infection. Whether regulation of chromatinisation of the viral genome is mediated by components of ND10 will also be examined. The possibility that such repressive factors may be involved in the establishment and maintenance of latency will also be examined. Finally, an analysis of whether promoters from other classes of HCMV genes are also regulated by chromatin structure will be described.

2. Materials and Methods

2.1 Materials

All reagents were purchased from Sigma-Aldrich and the United Kingdom (UK) unless otherwise stated. A list of abbreviations for materials used during the work described in this thesis is present in Appendix I.

2.2 Cell culture

2.2.1 Cell propagation and maintenance

Cells were grown in Eagle's Minimal Essential Medium (EMEM; Gibco, Invitrogen Corp.) supplemented with 10% (v/v) Fetal Calf Serum (FCS), 100U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine (EMEM-10) at 37°C in a humidified 5% CO₂ atmosphere unless otherwise stated. Cells were maintained as such: confluent monolayers in T175 flasks were passed every 3-4 days via pre-washing of cells with phosphate buffered saline (PBS) before incubation with 3ml of Trypsin-EDTA (0.5g Trypsin, 0.2g EDTA, 0.85g NaCl/L, Gibco) in 7ml of PBS at 37°C for 5 min. Cells were then split 1:3 into T175 flasks (Corning) with 40ml of fresh medium. To maintain low passage stocks of cells, upon receipt of the cell line, cells were grown to bulk. Cells were then trypsinised as above before centrifugation (465g, 5 min) and re-suspension of the pellet to approximately 2×10^6 cells per ml in filtered freezing medium (90% FCS, 10% DMSO). 1ml aliquots were then made in cryovials (Nunc) and stored at -80°C in cotton wool overnight before long term storage in liquid nitrogen.

Human foreskin fibroblasts (HFFs), fully permissive for HCMV infection, were obtained from the American Type Culture Collection (ATCC, USA), passed as above and were used for virus propagation (see 2.4.2). The passage number of the cells used ranged between 15-25 for all experiments.

Murine embryonic fibroblasts (MEFs), a kind gift of Dr Mike Gill (Division of Virology, University of Cambridge) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% fetal calf serum (DMEM-10). Medium for MEFs was further supplemented with 50µM 2-mercaptoethanol and cells were passed 1:3 every 3-4 days as above.

The human glioblastoma/astrocytoma cell line, U373-MG, obtained from the European Collection of Cell Cultures (ECACC) and fully permissive for infection, was also passed 1:4 every 4 days as above. U373-MG cells stably transfected with either pDsRed-N1 or pDsRed-N1-hDaxx plasmids (U373-dsRed and U373-dsDaxx respectively (Woodhall *et al.*, 2006)) were selected with G418 (1mg/ml; Invitrogen) supplemented medium and passaged as the parent cells.

The human embryonal carcinoma cell line, NT2D1 (referred to herein as T2 cells), was obtained from ATCC. T2 cells were passed as above 1:3 every 2-3 days and were differentiated to a fully permissive cell type (T2RA) by addition of 1 μ M retinoic acid (RA) in EMEM-10 for 5 days.

The human acute monocytic leukaemia cell line, THP1, was obtained from ATCC. As THP1s grow in suspension, cells were first centrifuged at 465g for 5 min before being split 1:3 into a new T175 with fresh RPMI-1640 medium (Gibco) supplemented as above (RPMI-10). Cells were differentiated to a macrophage-like (MØ) permissive cell type through treatment with 20nM phorbol 12-myristate 13-acetate (PMA) and 50 μ M hydrocortisone (HC) supplemented RPMI-10 overnight.

2.2.2 Trichostatin A (TSA) treatment of cells

All TSA (Upstate) and control media was created fresh on the day of experimentation. TSA medium was produced at a concentration of 330nM by dilution of TSA 1:3 with the solvent DMSO and then 1:1000 in EMEM-10. Control DMSO medium was produced by diluting the same volume of DMSO as used above 1:1000 into EMEM-10. Cells were then washed with 1ml of TSA or DMSO media before replacement with 2ml of fresh TSA or DMSO media for 16 hours. All virus infections were then carried out in the appropriate media.

2.2.3 Phosphonoformic acid (PFA) treatment of cells

All PFA medium was created fresh on the day of experimentation. A working stock of 10mg/ml PFA in dH₂O was diluted 1:100 into fresh EMEM-10. PFA media was then added to cells 24 hours prior to experimentation.

2.3 Monocyte isolation from peripheral blood and differentiation

2.3.1 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected via percutaneous venepuncture by a trained phlebotomist and anti-coagulated with heparin sulphate (10 units per 1ml blood; Addenbrooke's Hospital Pharmacy). The blood was then diluted 1:1 in sterile PBS before 35ml was layered onto 15ml of Lymphoprep (9.6% (w/v) sodium metrizoate, 5.6% (w/v) polysaccharide; Axis-Shield) and centrifuged for 20 min at 800g, with no brake, to separate the cell populations. After this time, the cloudy white band of mononuclear cells, formed between the plasma and Lymphoprep layers, was carefully retrieved using a plastic Pasteur pipette. The cells were then washed in sterile PBS via centrifugation (450g, 10 min) and the final pellet re-suspended in 5ml of PBS.

2.3.2 Isolation of monocytes from PBMC by adherence

Mononuclear cells isolated by density-dependent centrifugation were added to plastic 6-well plates (Nunc) in sterile PBS for 2 hours at 37°C in 5% CO₂. The monocytes, which are adherent, were then isolated by washing of the wells with sterile PBS to remove lymphocytes and cultured by the addition of X-Vivo 15 medium (Biowhittaker) supplemented with 10% human serum and 2mM L-glutamine.

2.3.3 Differentiation of monocytes to dendritic cells (DC)

Monocytes were first differentiated to immature DCs by the addition of interleukin-4 (IL-4, 1000U/ml; Peprotech) and granulocyte macrophage colony-stimulating factor (GM-CSF, 1000U/ml; Peprotech) to the culture X-Vivo 15 medium and the cells grown for 6 days at 37°C in 5% CO₂. Following incubation, the culture medium was further supplemented with lipopolysaccharide (LPS, 50ng/ml) for 3 days to induce full maturation.

2.4 Viruses

2.4.1 HCMV strains

Toledo is a commonly used low passage laboratory strain of HCMV. TB40/E (a kind gift of Christian Sinzger) is a low passage endothelial adapted clinical isolate that, although plaque purified, may contain up to three separate virus clones (Sinzger *et al.*, 2008). The AD169 strain (VR-538; ATCC) is a commercially available laboratory adapted strain of HCMV.

2.3 Monocyte isolation from peripheral blood and differentiation

2.3.1 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected via percutaneous venepuncture by a trained phlebotomist and anti-coagulated with heparin sulphate (10 units per 1ml blood; Addenbrooke's Hospital Pharmacy). The blood was then diluted 1:1 in sterile PBS before 35ml was layered onto 15ml of Lymphoprep (9.6% (w/v) sodium metrizoate, 5.6% (w/v) polysaccharide; Axis-Shield) and centrifuged for 20 min at 800g, with no brake, to separate the cell populations. After this time, the cloudy white band of mononuclear cells, formed between the plasma and Lymphoprep layers, was carefully retrieved using a plastic Pasteur pipette. The cells were then washed in sterile PBS via centrifugation (450g, 10 min) and the final pellet re-suspended in 5ml of PBS.

2.3.2 Isolation of monocytes from PBMC by adherence

Mononuclear cells isolated by density-dependent centrifugation were added to plastic 6-well plates (Nunc) in sterile PBS for 2 hours at 37°C in 5% CO₂. The monocytes, which are adherent, were then isolated by washing of the wells with sterile PBS to remove lymphocytes and cultured by the addition of X-Vivo 15 medium (Biowhittaker) supplemented with 10% human serum and 2mM L-glutamine.

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2.4.2 Virus propagation

Typically, between 10 and 20 T175 flasks of HFFs were infected at a multiplicity of infection (MOI) of 0.1 in 7ml of EMEM-10 for 3 hours at room temperature with gentle rocking. After this time, 18ml of EMEM-10 was added and the flask incubated at 37°C in a humidified 5% CO₂ atmosphere. Upon 100% infection, seen via cytopathic effect (CPE), the media was removed and stored at -80°C with 25 ml of fresh media then being added to the flask. This was then repeated every 2 to 3 days, with pooling of the media, until all cells had become detached from the flask.

Working stocks of HCMV were concentrated via centrifugation. Pooled frozen virus media was thawed and spun at 465g for 10 min at room temperature in an ALC PK 120 bench centrifuge to remove cellular debris. The supernatant was then taken and spun at 13,500g for 2 hours at 4°C in a Beckman Coulter Optima L-90K ultracentrifuge (rotor 19) to pellet the virus. The pellets were then re-suspended in an appropriate volume of EMEM-10 and aliquoted into 200µl samples and stored at -80°C unless stock was needed for gradient purification, in which case the pellet was re-suspended in 2ml of PBS and used as below (see 2.4.3).

2.4.3 Gradient purification of virus

2.4.3.1 Ficoll protocol

In two Ultra Clear 25mm tubes (Beckman Coulter), gradients were set up from 5ml of each of 15%, 12.5%, 10%, 7.5% and 5% Ficoll (w/v) made up in PBS. 1ml of the re-suspended clarified virus was then added to the surface of both gradients and the tubes spun at 19,000g for 2 hours at 4°C in a Optima L-90K ultracentrifuge (rotor SW28; Beckman Coulter) with no braking to separate the non-infectious enveloped particles (NIEPs), virions and dense bodies (DBs). Bands were then harvested using sterile plastic pastettes and pooled into three separate tubes. The pooled material was then made up to 30ml with PBS and spun again at 49,500g for 1 hour at 4°C (rotor SW41). The supernatant was then poured off before the pellets were re-suspended in 500µl of PBS, aliquoted into 50µl samples and stored at -80°C.

2.4.3.2 Sorbitol protocol

In two Ultra Clear 14mm tubes (Beckman Coulter), gradients were set up from 1.6ml of each of 70%, 60%, 50%, 40%, 30% and 20% sorbitol (w/v) made up in PBS. 1ml of the re-suspended clarified virus was then added to the surface of both gradients and the tubes spun at 49,500g for 1 hour at 4°C (rotor SW41) with no braking to separate the non-infectious enveloped particles (NIEPs), virions and dense bodies (DBs). Bands were then harvested using sterile plastic pastettes and pooled into three separate tubes. The pooled material was then made up to 10ml with PBS and spun again at 44,500g for 1 hour at 4°C (rotor SW41). The supernatant was then poured off before the pellets were re-suspended in 500µl of PBS, aliquoted into 50µl samples and stored at -80°C.

2.4.4 Titration of virus

2.4.4.1 Plaque assay

To titre the virus, 24-well plates were seeded to between 80-90% with HFFs. The virus was then serially diluted in EMEM-10 and 250µl added in duplicate to two wells for each dilution, before rocking the plate for 3 hours at room temperature. After this time, the virus dilution media was aspirated from the well and each well washed with 0.5ml of fresh media before replacement with 1ml of EMEM-10 containing γ -globulin (16µg/ml). Cells were then incubated at 37°C in a humidified 5% CO₂ atmosphere. After 14 days, cells were washed in PBS before fixation with 70% ethanol/dH₂O for 10 min at -20°C, washed again and then stained with Giemsa stain before counting of plaques.

2.4.4.2 TCID₅₀

Due to the duration of time necessary to carry out plaque assay analysis on HCMV preparations, a quicker endpoint dilution analysis was employed: the tissue culture infective dose 50% (TCID₅₀) analysis technique. HFFs are first re-suspended to 1×10^5 /ml in EMEM-10 and 180µl of the suspension aliquoted into each well of a 96-well plate. The virus being titred is then serially diluted 1:10 (20µl into each well) along the length of the 96-well plate in sextuplicate and the plate incubated at 37°C in a humidified 5% CO₂ atmosphere. After 10 days, the plates were washed in PBS before fixation with 70% ethanol/dH₂O for 10 min at -20°C, washed again and then stained with Giemsa stain. It was then determined for each dilution where the endpoint dilution had occurred and the following calculation used to determine the TCID₅₀ of the virus:

$$\log_{10} \text{TCID}_{50} = A - [B \times (C - 0.5)]$$

where:

A = the last index value of 100% infection

B = the log of the dilution factor (i.e. $\log(10) = 1$)

C = the sum of the percentage of infected wells

The TCID_{50} per ml of virus is then determined before plaque forming units (PFU) per ml of virus is calculated by the following equation:

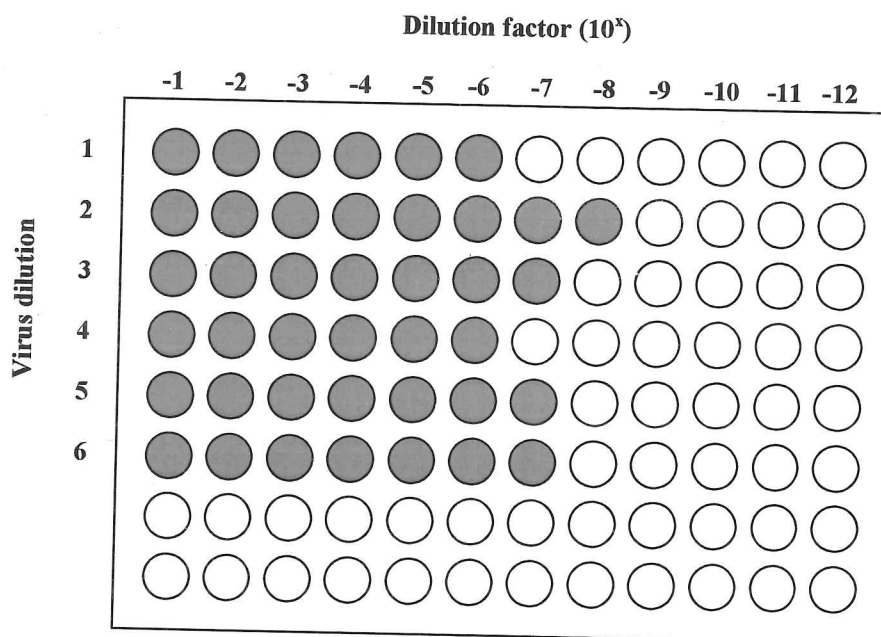
$$\text{PFU/ml} = \text{TCID}_{50}/\text{ml} \times 0.69$$

For a full example of the TCID_{50} method, see Fig. 2.1.

2.5 Western blot analysis of protein expression

2.5.1 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing SDS-PAGE was carried out as follows. Cellular protein samples were prepared such that 10^5 cells were re-suspended in 20 μl of Laemmli's loading buffer (0.125M Tris-HCl, pH 6.8; 25% (v/v) glycerol; 1.25% (v/v) 2-Mercaptoethanol; 1.25% SDS; 72.5% (v/v) dH_2O). Electrophoresis of samples was typically performed on a 10% Bis-acrylamide resolving gel (10ml Bis-acrylamide [0.8:30]; 3.75ml 3M Tris, pH 8.8; 0.3ml 10% SDS; 16.95ml dH_2O ; 15 μl TEMED; 1.5ml 1.5% ammonium persulphate) overlaid with 0.5ml H_2O -saturated butan-2-ol. After polymerisation had occurred, H_2O was used to wash the surface of the resolving gel before the stacking gel was added (2.5ml Bis-acrylamide [0.8:30]; 5ml 0.5M Tris, pH 6.8; 0.2ml 10% SDS; 11.3ml dH_2O ; 15 μl TEMED; 1ml 1.5% ammonium persulphate). After loading of 20 μl samples, electrophoresis was carried out at 100V for 1-2 hours in SDS running buffer (3g/l Tris-base; 14.4g/l glycine; 1g/l SDS; dH_2O to 1 litre). Transfer to a nitrocellulose membrane was then performed overnight at $+4^\circ\text{C}$ in 20% transfer buffer (1g/l Tris-base; 4.8g/l glycine; 20% methanol; dH_2O to 1 litre) at 20V. Protein transfer and size was checked by the use of rainbow markers (Amersham Biosciences). If necessary, as a loading control, a duplicate SDS-PAGE gel was rocked in coomassie stain (10% acetic acid; 20% ethanol; 70% dH_2O) for 30 mins before being washed in destain (10% acetic acid; 20% ethanol; 69.9% dH_2O ; 0.1% w/v brilliant blue)



Example:

Dilution 10^{-6} has all (6/6) wells infected = 1

Dilution 10^{-7} has 4/6 wells infected = 0.66

Dilution 10^{-8} has 1/6 wells infected = 0.16

Dilution 10^{-9} has 0/6 wells infected = 0

$$\log_{10} \text{TCID}_{50} = A - [B \times (C - 0.5)]$$

Where:

A = the last index value of 100% infection, i.e. -6

B = the log of the dilution factor, i.e. $\log(10) = 1$

C = the sum of the percentage of infected wells, i.e. $1 + 0.66 + 0.16 = 1.82$

$$\begin{aligned} \log_{10} \text{TCID}_{50} &= -6 - [1 \times (1.82 - 0.5)] \\ &= -7.32 \end{aligned}$$

$$\begin{aligned} \text{TCID}_{50} &= 10^{-7.82} \\ &= 1 / 10^{7.82} \\ &= 1 / 2.1 \times 10^7 \text{ in } 20\mu\text{l} \\ &= 1 / 1.0 \times 10^9 \text{ in } 1\text{ml} \end{aligned}$$

Therefore, the 50% end point titre is 1.0×10^9

$$\begin{aligned} \text{PFU/ml} &= 1.0 \times 10^9 \times 0.69 \\ &= 7.2 \times 10^8 \end{aligned}$$

Figure 2.1 The TCID_{50} calculation. Quantitation of the concentration of virus in a sample by TDID_{50} and the resultant PFU determination was carried out as shown in the example above.

until clearly separated protein bands could be seen. Images were created using a Pulnix TM-300 imager.

2.5.2 Western blot analysis

Following transfer of SDS-PAGE separated protein samples, nitrocellulose membranes were pre-treated in blocking solution, 5% dried milk (w/v) (Marvel; Chivers, Ireland) in PBS, for 30 min before antibody incubations. Primary antibody incubation was carried out in blocking solution for 60 min at room temperature at a concentration determined individually for each antibody (see Table 2.1). Primary antibodies were detected using horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG antiserum (Santa Cruz, USA) or HRP-conjugated rabbit anti-mouse IgG antiserum (DAKO) diluted 1:2,000 in blocking solution, depending upon the primary isotype, for 30 min at room temperature. All antibody incubations were followed by four 15 min washes of the nitrocellulose membrane in PBS/0.1% Tween-20. Detection of protein presence was performed using Enhanced Chemiluminescence (ECL, Amersham) by mixing equal quantities, typically 500 μ l, of solutions A and B before addition to each membrane for 1 min at room temperature with slight agitation. Excess ECL reagents were drained from the membrane, which was then placed in Saran wrap before exposure to autoradiographic film to quantify protein levels.

2.6 Immunofluorescence analysis of protein expression

2.6.1 Immunofluorescence (IF)

Typically, cells were seeded at 1×10^5 per well in 8 well glass chamber slides. Cells were then infected and/or transfected appropriately depending on the specific experiment before incubation at 37°C in a humidified 5% CO₂ atmosphere for a specified time. Media was then removed and, if using T2 cells, cells were then fixed in 4% paraformaldehyde for 10 min at room temperature. All cells were then washed with PBS before the addition of 70% ethanol/dH₂O for 30 min at -20°C, as all other cell types were treated, for fixation and permeabilisation, unless permeabilisation was not needed for T2/T2RA surface staining. After the slides were subjected to four 10 min washes in PBS/1% FCS, primary antibodies were added appropriately diluted in wash solution (see Table 2.1). Then, after a further round of washes, secondary FITC, TRITC or Alexa 594 conjugated antibodies were added as necessary at a dilution in wash solution determined for each, along with Hoechst 33342

Primary antibodies

Antibody	Manu. #	Manu.	WB	IF	ChIP	Host
GAPDH (HRP-conj)	ab9385	Abcam	1:2000	-	-	Rabbit
hDaxx	D7810	Sigma	1:4000	-	-	Rabbit
hDaxx	07-471	Upstate	-	1:200	-	Rabbit
Histone H3, CT, pan	07-690	Upstate	-	-	1:200	Rabbit
Histone H3, CT, pan	14-411	Upstate	1:5000	-	-	Rabbit
Histone H3 dimethyl lys4	07-030	Upstate	-	-	1:200	Rabbit
Histone H3 dimethyl lys9	07-441	Upstate	1:2000	-	1:200	Rabbit
Histone H4 acetylated	06-866	Upstate	1:2000	-	1:200	Rabbit
HP1 β (M31)	MCA1946	Serotec	1:2000	1:100	1:200	Rat
IE72/86 (E13)	11-003	Argene	1:1200	1:100	-	Mouse
mDaxx (M-112)	sc-7152	Santa Cruz	1:1000	-	-	Rabbit
Mouse serum	M-5905	Sigma	-	-	1:200	Mouse
Oct4	ab19857	Abcam	1:2000	1:100	-	Rabbit
PML	P6746	Sigma	1:2000	-	-	Mouse
PML (N-19)	sc-9862	Santa Cruz	-	1:100	-	Goat
pp28	ab6502	Abcam	1:5000	-	-	Mouse
Rabbit serum	R-9133	Sigma	-	-	1:200	Rabbit
Rat serum	R-9759	Sigma	-	-	1:200	Rat
SSEA-3	ab16286	Abcam	-	1:100	-	Rat
UL44 (p52)	M0757	Dako	1:1000	-	-	Mouse

Secondary antibodies

Antibody	Manu. #	Manu.	WB	IF	Host
Anti-goat FITC	F2016	Sigma	-	1:30	Rabbit
Anti-goat TRITC	T-7028	Sigma	-	1:30	Rabbit
Anti-mouse FITC	F3008	Sigma	-	1:30	Sheep
Anti-mouse HRP-conj	P0260	DAKO	1:2000	-	Rabbit
Anti-mouse IgG Alexa 594	A21203	Invitrogen	-	1:100	Donkey
Anti-mouse TRITC	T-5393	Sigma	-	1:30	Goat
Anti-rabbit FITC	F1262	Sigma	-	1:30	Goat
Anti-rabbit HRP-conj	sc-2004	Santa Cruz	1:2000	-	Goat
Anti-rabbit TRITC	T-5268	Sigma	-	1:30	Goat
Anti-rat HRP-conj	P0450	DAKO	1:2000	-	Rabbit
Anti-rat IgM Alexa 594	A21213	Invitrogen	-	1:100	Goat

Table 2.1 Primary and secondary antibodies and conditions of use. Listed are the primary and secondary antibodies used within this study. All appropriate dilutions are given for Western blot (WB), immunofluorescence (IF) and Chromatin immunoprecipitation (ChIP).

(1µg/ml) to counter stain nuclei, for 45 min before a final round of washes. Slides were then viewed using a Nikon Eclipse TE300 UV microscope and Hamamatsu colour chilled 3CCD camera.

2.6.2 Fluorescence activated cell sorting (FACS)

2.6.2.1 FACS of infected fixed cells

HFFs were seeded in 6 well plates at 1×10^6 per well. After 24 hours, media was aspirated from the monolayers and replaced with 0.5ml of pre-diluted virus mix (MOI 0.1) in EMEM-10 with incubation for 3hr at 37°C in a humidified 5% CO₂ atmosphere. The viral media was then removed and monolayers washed before replacement with fresh EMEM-10 and overnight incubation. After a further 24 hours, cells were removed from wells via trypsinisation and pelleted (465g, 3 min) in FACS tubes before washing with 1ml of PBS. Cells were then fixed with 70% ethanol/dH₂O for 30 min at -20°C. After pelleting, cells were then washed as above and stained as follows: 50µl of primary antibody, diluted as appropriate in PBS/1% FCS, was added to the pellet and the cells re-suspended and incubated for 1 hour. Cells were stained for the presence of IE protein expression with the anti-IE72/86 antibody (E13, Argene) diluted 1:100. After this time, the cells were pelleted and washed twice in PBS before addition of secondary anti-mouse FITC antibody diluted 1:30 in PBS/1% FCS for 45 min. Finally, the cells were washed twice again as above before resuspension of the final pellet in 0.5ml of 1% formaldehyde/PBS. Analysis of the cells was then carried out on a Becton Dickinson FACSort machine and then the data processed using WinMDI 2.8 flow cytometry application.

2.6.2.2 FACS of live cells

All sorting of live cells took place on a MoFlo High-Performance cell sorter (Dako) operated by Richard Grenfell at the Laboratory of Molecular Biology (LMB, University of Cambridge). A 528nm argon ion laser was used to detect dsRed-construct stably transfected U373s. Cells were re-suspended in fresh EMEM-10 before sorting through the disinfected machine and collected into 2ml of EMEM-10. This volume was then used to seed 1 well of a 6-well plate, the cells were allowed to grow and finally bulked up as necessary.

2.7 Chromatin Immunoprecipitation (ChIP) assay

Cells were typically seeded at 1×10^6 per well on 6 well plates and after 24 hours were synchronised in serum-free media for 48 hours before infection with HCMV at a low MOI (0.1). Additionally, in order to exclusively analyse input genomes, cells were pre-treated with PFA (100 μ g/ml) for 24 hours prior to infection with HCMV to inhibit replication of viral DNA. At the necessary time point, cells and chromatin were fixed via the addition of neat 40% formaldehyde (1:40 dilution) for 10 min at room temperature (Fig. 2.2). Monolayers were then washed with PBS before replacement with 1ml of PBS, which the cells were then scraped into for harvesting. After pelleting (465g, 5 min), cells were lysed in 200 μ l of lysis buffer (1% SDS; 10mM EDTA; 50mM Tris, pH 8.1; 1mM PMSF; 1mM pepstatin A; 1mM aproprotin) before sonication for 50 seconds (setting 5; Heat Systems sonicator XL). Cell debris was then removed by centrifugation of the samples at 4,000g for 5 min. The supernatant was then made up to 2ml with dilution buffer (0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris, pH 8.1; 150mM NaCl), with 200 μ l being removed and added to 300 μ l of elution buffer (1% SDS; 0.1M NaHCO₃) as an input (10%) sample, before addition of 160 μ l of protein A sepharose bead slurry (beads were pre-swollen in PBS and supplemented with 200 μ g/ml of sheared herring sperm DNA) and rotation of tubes at 4°C for 30 min to pre-clear the sample. Samples were then spun (4000g, 5 min) to pellet the beads and the supernatant aliquoted equally into separate tubes before addition of the appropriate antibodies. Chromatin was precipitated using anti-pan histone-H3 (panH3), anti-acetylated histone H4 (H4ac), anti-dimethyl lysine 4 histone H3 (H3K4me2), anti-dimethyl lysine 9 histone H3 (H3K9me2) and anti-HP1 β antibodies or IgG normal serum controls, as detailed for each experiment. Immunoprecipitation was carried out with rotation at 4°C overnight.

Following incubation, 60 μ l of protein A sepharose bead slurry was added to the samples and each rotated at 4°C for a further 1 hour. Samples were then spun (16,000g, 5 min) and the supernatant removed to waste before the beads were washed with 0.5ml of dilution buffer. This process was then repeated using wash buffer 2 (0.1% SDS; 1% Triton X100; 2mM EDTA; 20mM Tris, pH 8.1; 500mM NaCl), wash buffer 3 (0.25M LiCl; 1% NP40; 1% sodium deoxycholate; 1mM EDTA; 10mM Tris, pH 8.1) and then two Tris-EDTA buffer washes. The protein complexes were then eluted from the beads with two 15 min rotating washes in 250 μ l of elution buffer at room temperature, retaining the supernatant

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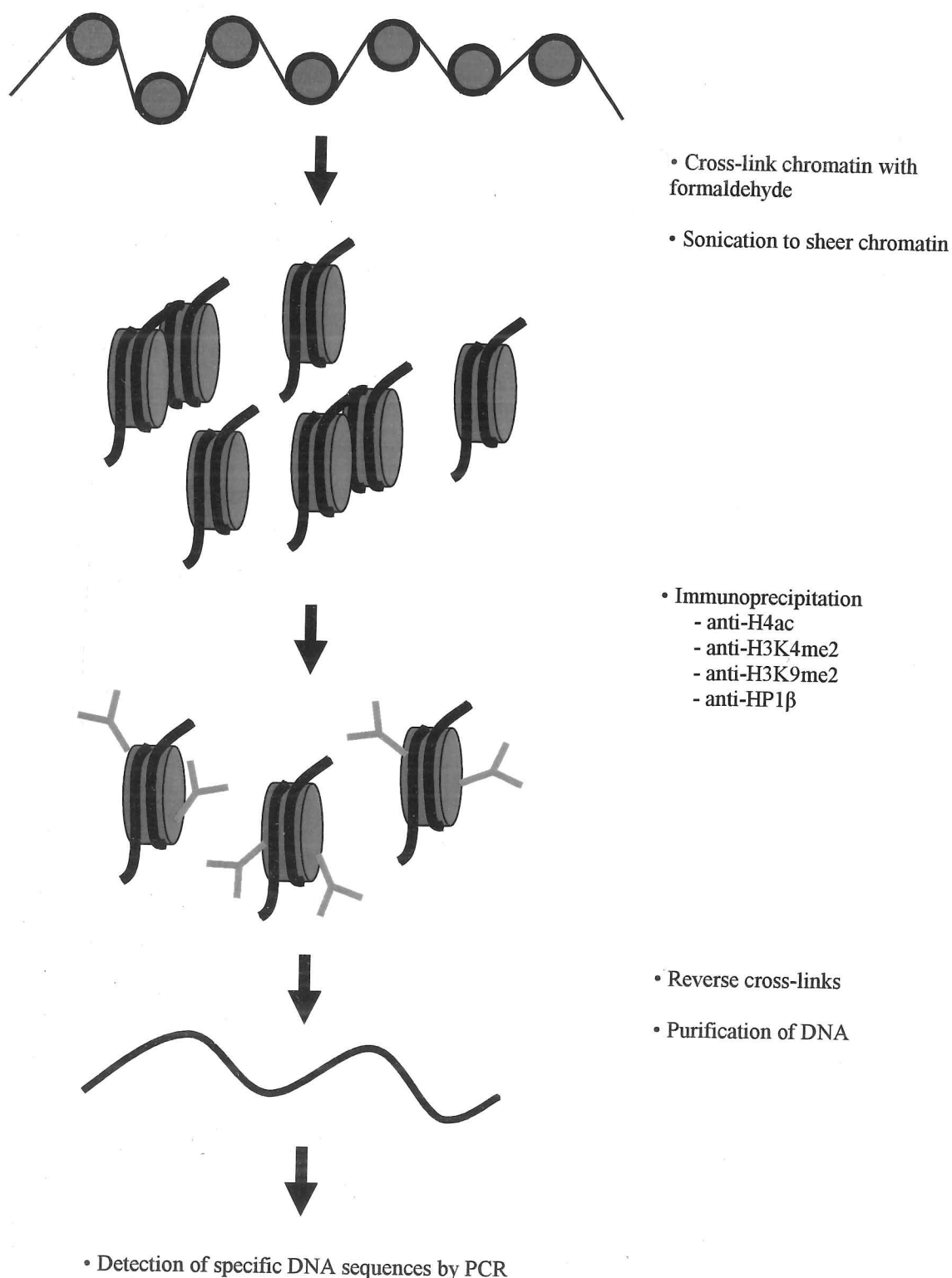


Figure 2.2 Chromatin immunoprecipitation (ChIP) assay. Cells are subjected to 1% formaldehyde for 15 min to fix protein:DNA interactions. The cells are then lysed and the sample sonicated to separate nucleosomes before incubation with specific antibodies. The primary antibody is then recovered using protein A sepharose beads and elution before the nascent DNA from the nucleosomes is isolated first by reversing the cross-links with NaCl and the DNA acquired by standard phenol/chloroform extraction. Purified DNA is precipitated in isopropanol and then can be analysed using specific PCR to determine the presence or absence of specific sequences.

from the pelleted beads each time. To each 500 μ l sample, 20 μ l of 5M NaCl was added before being incubated at 65°C for 4 hours. Then, a further 10 μ l of 0.5M EDTA, 40 μ l of 0.5M Tris (pH 6.8) and 2 μ l of proteinase K (10mg/ml in dH₂O) was added before incubation at 42°C for 1 hour. After this time, 1ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample and shaken for 2 min before being centrifuged (16,000g, 15 min) to extract the DNA. The upper aqueous solution was then added to an equal volume of isopropanol, shaken and then placed at -20°C overnight. Precipitated DNA was pelleted by centrifugation (16,000g, 30 min) and then re-suspended in TE buffer, typically in 0.5ml for inputs and 50 μ l for samples. Promoter specific PCR was then set up as detailed below using the same volume of input DNA as sample DNA in each reaction. In general, the volume used was 5 μ l so that 0.1% of total chromatin was used as an input and for ChIP samples the immunoprecipitated proportion from around 2-3% of starting total chromatin. Thus, although the amount of input DNA used for PCR was around 20-30 fold less than possible immunoprecipitated DNA, due to the efficiency of the ChIP assay this meant that PCR would provide sample data equivalent with input being set as 1 in the quantitative analysis when association of a modified histone was present.

2.8 Polymerase chain reaction (PCR)

2.8.1 Semi-quantitative PCR

PCR DNA amplification was performed using the BioMix Red reaction mix (Bioline) containing BioTaq Red DNA polymerase, dNTPs and MgCl₂ (1.5mM). The user simply adds dH₂O, template and primers, and can increase the MgCl₂ concentration as required (for primers and MgCl₂ concentration see Table 2.2). Therefore, PCR was performed using the following mix per reaction (final concentrations of constituents given):

5 μ l DNA template
25 μ l 2x BioMix Red
1 μ l sense primer (1 μ M)
1 μ l antisense primer (1 μ M)
0.5-1.5 μ l MgCl₂ (2-3mM) as necessary
dH₂O up to 50 μ l

Target	Promoter or mRNA	Sense primer (5'-3')	Antisense primer (5'-3')	Annealing temp. (°C)	MgCl ₂ conc. (mM)	Product size (bp)	Reference*
c-Fos	Promoter	GAGACCTCTGAGACAGGAAC	CAGATGCGGTTGGAGTAC	55	3.0	230	Nevels <i>et al.</i> , (2004b)
γ-globin	Promoter	GCCTTGACCAATAGCCTTGACA	GAAATGACCCATGGCGTCTG	55	1.5	186	Bottardi <i>et al.</i> , (2003)
MIEP	Promoter	TGGGACTTTCCTACTTGG	CCAGGCGATCTGACGGTT	50	1.5	285	Murphy <i>et al.</i> , (2002)
MIEP [†]	Promoter	ATTACCATGGTGATGCGGTT	GGCGGAGTTGTTACGACAT	50	1.5	156	Reeves <i>et al.</i> , (2005b)
β2.7	RT-PCR	CGTTATCCGTTCCCTCGTAGG	GTTTCGTTGTTGTCGGTAGT	55	2.5	302	Reeves <i>et al.</i> , (2007)
GAPDH	mRNA	GAGTCAACGGATTGGTCTGT	TTGATTTTGGAGGGATCTCG	60	2.5	236	Yu <i>et al.</i> , (2005)
hDaxx	mRNA	GACGGACATTTCTCTTCCA	TCTCATGCACTGACCTTTGC	55	2.5	224	Hofmann <i>et al.</i> , (2002)
HP1β	mRNA	GTGGTAAAGGGCAAAGTGGA	CCTCGTGGCTTTTCTGACTC	55	2.5	245	
IE	mRNA	CGTCCTTGACACGATGGAGT	ATTCTTCGGCCAACTCTGGA	55	2.5	194	Reeves <i>et al.</i> , (2005b)
Oct4	mRNA	GCATACTGTGGACCTCAGG	CCAAGGTGATCCTCTTCTG	55	2.5	262	Park <i>et al.</i> , (2007a)
PML	mRNA	TCCCCTTCAGCTTCTCTTCA	CGCAGAACTGGAACCTCTC	55	2.5	232	

*Unless reference given, primers designed using Primer 7 software.

[†]PCR primers internal to MIEP primers and product used as a probe for Southern blot analysis

RTQ-PCR Target (promoter)	TM or SYBR Green	Sense primer (5'-3')	Antisense primer (5'-3')	TaqMan probe (5'-FAM to 3'-TMR)	MgCl ₂ conc. (mM)	Product size (bp)	Reference*
γ-globin	TM	GCCTTGACCAATAGCCTTGACA	GAAATGACCCATGGCGTCTG	ACCTCAGACGTTCCAGAAGCGAGTGT	4.5	186	Bottardi <i>et al.</i> , (2003)
GAPDH	TM	CGGCTACTAGCGGTTTACG	AAGAAGATGCGGCTGACTGT	CACGTAGCTCAGGCCTCAAGACCT	5.5	189	Coleman <i>et al.</i> , (2008)
MIEP	TM	CCAAGTCTCCACCCATTGAC	GACATTTTGGAAAGTCCCGTTG	TGGGAGTTTGTGTTTGGCACCAAA	4.5	71	Groves & Sinclair (2007)
pp28	SG	TGTTGCAGTCACACCCGGTGC	TTAGCGTGGCCCTGAAGAGC	N/A	2.5	112	
UL44	SG	AACCTGAGCGTGTTTGTG	CGTGCAAGTCTCGACTAAG	N/A	2.5	275	Nevels <i>et al.</i> , (2004b)

*Unless reference given, primers designed using Primer 7 software.

Table 2.2 & 2.3 PCR and RTQ-PCR primer sequences and conditions of use.

DNA amplification then took place using the following parameters: 5 min at 95°C (initial denaturation of template), then between 20-60 cycles of 40 sec at 94°C (separation of template), 40 sec at 50-60°C (primer annealing – temperature set for individual primer reactions), and 90 sec at 72°C (primer extension). After the final extension step, the PCR samples were then cooled to 4°C. Due to the presence of a red dye and density of the reaction mix, it was unnecessary to add any further loading buffer. Therefore, 20µl of each sample was separated in a 2% agarose gel, with ethidium bromide (10ng per gel), at 100V in TBE running buffer (40mM Tris-base; 90mM boric acid; 1mM EDTA, pH 8.0). To initially quantify DNA levels, bands were then visualised by UV light and Image J analysis of band intensity used to semi-quantify the relative levels of PCR products. For more specific analysis, Southern blot analysis was employed (see 2.9).

2.8.2 Real-time quantitative-PCR (RTQ-PCR)

2.8.2.1 TaqMan RTQ-PCR

During the course of the work presented in this thesis a RTQ-PCR assay was developed to enable more quantitative analysis of samples prepared from HCMV DNA. This development was carried out with the very kind help of Dr Heather Coleman (Division of Virology, University of Cambridge).

TIB MolBiol (Berlin, Germany) was approached to design a TaqMan assay that would specifically detect the MIEP when used on a Rotor-Gene 3000 real-time PCR machine (Corbett Research). TaqMan analysis uses primers usually placed no more than 100bp apart and a probe sequence that anneals between the sites of the primers (see Fig. 2.3). The probe is manufactured so that a fluorescent reporter and a fluorescence quencher are attached to either end of the RNA probe: in this conformation only background fluorescence is visible by the detection filters. Upon initiation of PCR, the polymerase begins copying the complementary strand 5' to 3' but whilst doing so must displace and cleave the RNA probe. This is done via cleavage and leads to the dissociation of the fluorescent reporter from the close proximity to the quencher, and thus allows the reporter to fluoresce. This output is detected in real-time by excitation and detection from each reaction at least every cycle of the PCR run. Furthermore, the final analysis is termed fully quantitative due to serial 10 fold dilutions of standards of known copy number being used

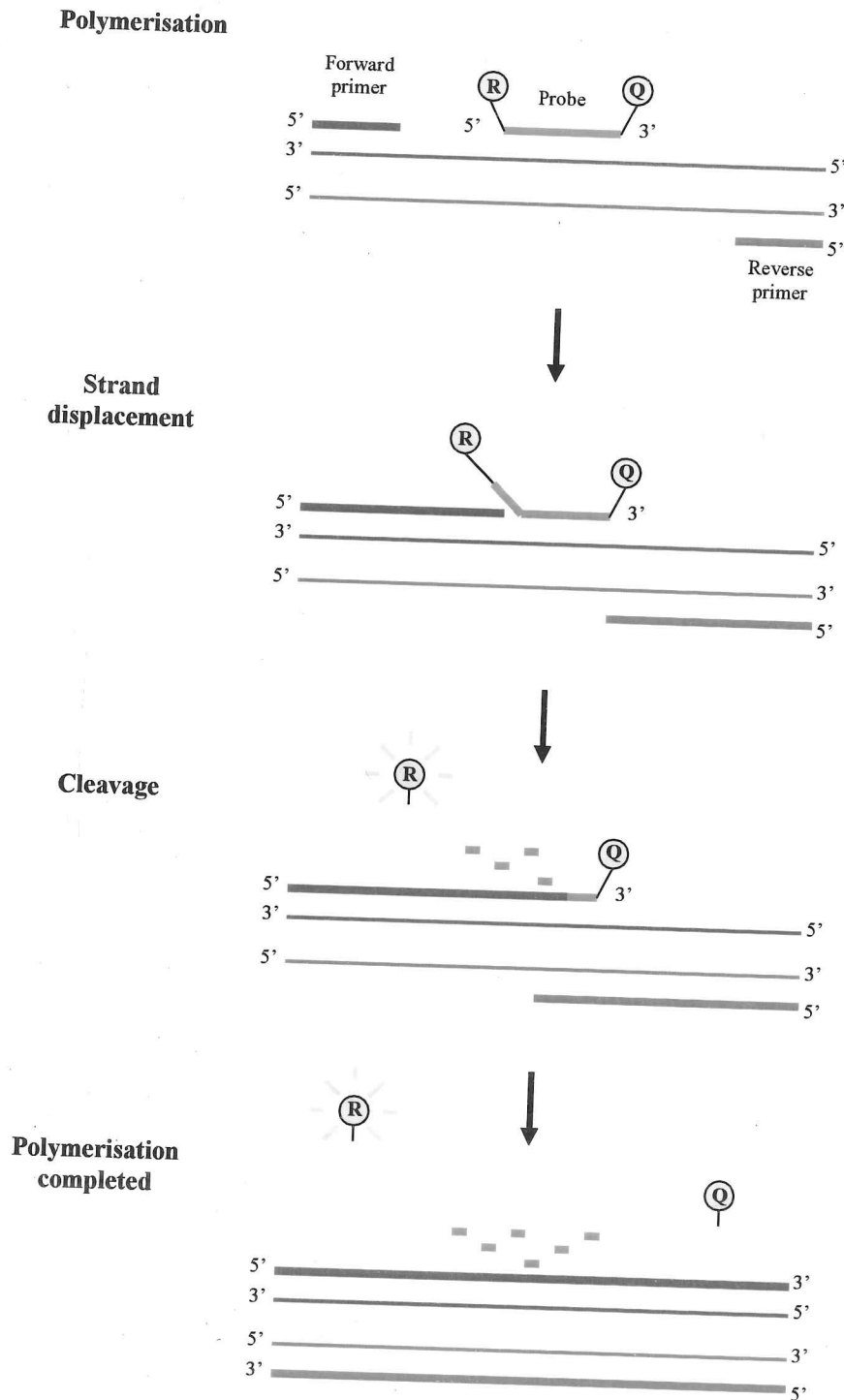


Figure 2.3 TaqMan real-time quantitative polymerase chain reaction (RTQ-PCR) analysis. The primers and probe first anneal to the denatured template DNA: one set per template strand. The RNA probe is composed of a fluorescent reporter (R) and a fluorescence quencher (Q) at either end of the RNA probe so that, until separation, the fluorescence is undetectable. Upon initiation, the polymerase progresses 5' to 3' down the template DNA and meets the probe where it is first displaced before being cleaved by the polymerase's nuclease activity. Due to release of the reporter from the close proximity to the quencher, fluorescence is now detectable and after completion of polymerisation and complete dissociation of reporter and quencher the fluorescent output can then be analysed quantitatively.

throughout the run to produce a standard curve. Hence, the technique is termed real-time quantitative PCR (RTQ-PCR).

The TaqMan primers and probe were designed such that they would anneal between the sites of primers already used for MIEP PCR: +13 to -272 relative to the MIEP transcription start site (see Table 2.3). In addition, the primers and probe were designed to work on all strains of HCMV with published genome sequences used routinely within the laboratory. The outcome were a sense and antisense primer that annealed complementary to positions -157 and -86 relative to the MIEP start site and a TaqMan probe with a 5'-6-carboxyfluorescein (5'-FAM) fluorescent reporter and a 3'-6-carboxy-tetramethyl-rhodamine (3'-TMR) quenching element (see Fig. 2.4 for positions on sequence). The standard used for the MIEP RTQ-PCR was the pEScat plasmid (see 2.13.5).

To preclude any contamination of the PCR reactions before analysis, all mixes other than including the template DNA were set up in a fully clean, uncontaminated PCR safe laboratory (room #5537, Dept. of Medicine). The master mix was composed of fully PCR safe reagents: 10x PCR buffer, $MgCl_2$ (to the necessary concentration) and HotStarTaq polymerase (Qiagen), 1x dNTPs (Bioline), dH_2O and the primers and probe (see Table 2.4 for full details). This mix was then removed from the PCR safe laboratory and 12 μ l aliquoted into each 0.1ml tube (Corbett Research) before addition of 8 μ l of DNA template, usually in triplicate. The tubes were then assembled into the rotor and the machine run for 45 cycles as so: 95°C for 15 min (polymerase activation and template dissociation), 60°C for 60 sec (primer annealing and extension) and 95°C for 15 sec (separation of template). The fluorescence output was taken every cycle and the copy number in each sample calculated by the Rotor-Gene 3000 software (Rotor-Gene 6; Corbett Research) against the control standard curve (for an example see Fig. 6.3b).

In addition to using TaqMan RTQ-PCR for analysis of the viral MIEP, this method was also used to quantitatively analyse the copy number of cellular human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and γ -globin sequences within a DNA sample. The TaqMan primers and probes were designed by TIB MolBiol and supplied by Heather Coleman. For further details of primer/probe design and reaction setup, see Tables 2.3 and

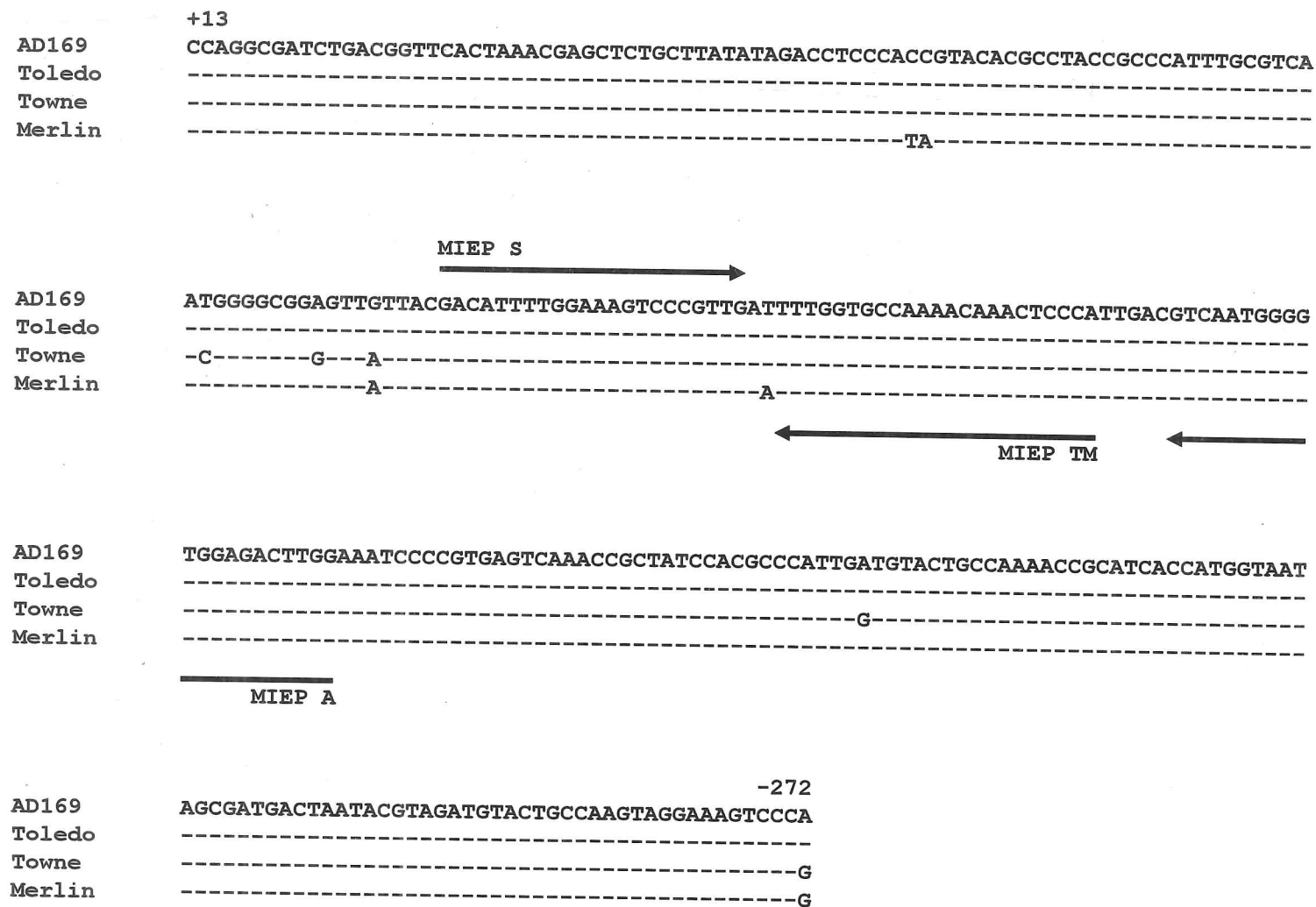


Figure 2.4 MIEP RTQ-PCR primer sites. The figure shows the sites of the two primers, sense (S) and antisense (A), and the TaqMan probe (TM) for the MIEP specific RTQ-PCR analysis positioned along the aligned MIEP sequences of four strains (AD169, Toledo, Towne and Merlin) of HCMV from +13 to -272 relative to the MIEP transcription start site.

2.4 respectively. Plasmid standards were also supplied (pGEM-T-Easy- γ globin and pGEM-T-Easy-GAPDH respectively; see 2.13.8 and 2.13.9).

2.8.2.2 SYBR Green RTQ-PCR

In addition to the use of a TaqMan primers and probe set, the use of SYBR Green to quantitatively analyse DNA samples during RTQ-PCR is also available. Here, instead of a fluorescent RNA probe, SYBR Green specifically intercalates into dsDNA and is only able to fluoresce once this has occurred. Therefore, the fluorescence emitted by SYBR Green upon every cycle can be used to quantify the copy number of a starting DNA template when compared to a known standard curve. Furthermore, SYBR Green is readily available as a concentrated mix with polymerase, the necessary buffers, MgCl_2 (2.5mM, unless concentration change was necessary) and dNTPs (Qiagen). Thus, the user simply adds specific primers and DNA template for each reaction. The master mix was again setup in the PCR safe laboratory (10 μ l of 2x SYBR Green, 1 μ l of sense primer and 1 μ l of antisense primer per reaction; see Table 2.4) before removal and 12 μ l aliquoted into each 0.1ml tube before addition of 8 μ l of DNA template, usually in triplicate. The tubes were then loaded into the rotor (Rotor-Gene 3000) and the samples amplified for 45 cycles as follows: 95°C for 15 min (polymerase activation and template denaturation), 55°C for 20 sec (primer annealing), 72°C for 20 sec (primer extension) and 95°C for 5 sec (denaturation). Readings were taken every cycle, as above, and the copy number determined via calculation dependent on a known standard curve. SYBR Green RTQ-PCR was used to analyse the copy number of viral early and late promoters, UL44 and pp28 respectively, and a 10 fold serial dilution of promoter standard plasmids used (see 2.13.2 and 2.13.3).

2.9 Southern blot analysis

2.9.1 Southern blot

Specific analysis of DNA levels produced by PCR using the BioMix Red reaction mix was achieved by using the Southern blot technique and hybridisation of DNA with a specific radio-labelled probe. After electrophoresis, the gel was washed in 0.25M HCl for 20 mins before a further wash in 0.4M NaOH for 20 min. DNA was then transferred overnight to nitrocellulose (Hybond N⁺, Amersham) via capillary action from the membrane-overlaid gel with residual 0.4M NaOH. Following transfer, the nitrocellulose membrane was washed in 5x SSC (20x SSC: 3M NaCl; 1M sodium citrate, pH 7.0) and incubated with

Reagent	γ globin	GAPDH	MIEP	pp28	UL44
2x SYBR Green				10	10
10x buffer	2	2	2		
MgCl ₂	2.4	3.2	2.4		
dNTPs	1.6	1.6	1.6		
Sense primer	1.8	0.6	0.6	1	1
Antisense primer	0.6	1.8	0.1	1	1
TaqMan probe	0.1	0.1	0.05		
HotStarTaq	0.16	0.16	0.16		
H ₂ O	2.74	2.54	5.09		
DNA template	8	8	8	8	8
Total	20	20	20	20	20

Table 2.4 TaqMan & SYBR Green RTQ-PCR reaction setup.

15ml of pre-hybridisation buffer (10ml 50x Denhardt's solution [5g polyvinylpyrrolidone; 5g BSA; 5g Ficoll; up to 500ml dH₂O]; 30ml 20x SSC; 0.5ml 20% SDS; 54.5ml dH₂O) supplemented with 0.5ml of 10mg/ml sheared herring sperm DNA in a Teche hybridisation tube for 1 hour at 62°C. After this time, a pre-boiled aliquot of specific radio-labelled probe was added to the tube and the membrane incubated overnight at 62°C. After 24 hours, the membrane was washed twice in 50ml of 2x SSC/0.1% SDS for 30 min at 62°C and then twice in 50ml of a high stringency 0.2x SSC/0.1% SDS wash for 30 min at 62°C. The nitrocellulose membrane was then covered in Saran wrap and exposed to autoradiographic film.

2.9.2 Probe manufacture

DNA probes were produced via PCR from Toledo DNA using primers internally positioned compared to normal PCR amplification primers (see Table 2.2). The reaction products were column purified (Nucleobond) before being used to produce radio-labelled probes. Here, 25ng of probe DNA was made up to a volume of 45µl with dH₂O and then denatured at 95°C for 5 min. This was then added to pre-aliquoted Rediprime II mix (Amersham) before being mixed with the addition of 1.85MBq of ³²P-labelled αdCTP and incubated at room temperature for 1 hour. Purification of the probe was performed using a G50 Quickspin column (Boehringer Mannheim), which was pre-spun at 830g for 3 min. The Rediprime mix was then added to the centre of the column before re-spinning at 830g for 3 min. Incorporation of the radionucleotide was estimated by comparing the level of radioactivity present within the eluant (labelled probe) to the column (unincorporated radionucleotide). The probe was then aliquoted into 0.2MBq lots and stored at -20°C.

2.10 Transfection protocols

2.10.1 Lipofectamine transfection of siRNA oligonucleotides

Cells were seeded 24 hours prior to requirement. Approximately 4 hours before transfection, medium was removed from cells and replaced with Opti-MEM (Gibco) serum free transfection medium before incubation at 37°C in a humidified 5% CO₂ atmosphere. For every 10⁵ cells, 1µl of siRNA oligonucleotide (20µM) was added to 49µl of Opti-MEM and mixed gently. Separately, 0.5µl of Lipofectamine-2000 (Invitrogen) was added to 49.5µl of Opti-MEM and gently mixed. After 5 min, the Lipofectamine mixture was added to that of the siRNA and then incubated at room temperature for 20 min. Media was

then removed from the cells and 100 μ l of siRNA/Lipofectamine mix added to the monolayer before incubation at 37°C in a humidified 5% CO₂ atmosphere. After a further 4 hours, the cells were re-fed with EMEM-10. The cells were then used for investigation after 72 hours.

2.10.2 Lipofectamine transfection of plasmids

Transfection of plasmid DNA was carried out essentially as described above (2.10.1). Approximately 4 hours before transfection, medium was removed from cells and replaced with Opti-MEM serum free transfection medium before incubation at 37°C in a humidified 5% CO₂ atmosphere. For every 10⁵ cells, 0.4 μ l of plasmid (1 μ g/ μ l) was added to 49.6 μ l of Opti-MEM and mixed gently. Separately, 1 μ l of Lipofectamine-2000 was added to 49 μ l of Opti-MEM and gently mixed. After 5 min, the Lipofectamine mixture was added to that of the plasmid and then incubated at room temperature for 20 min. Media was then removed from the cells and 100 μ l of plasmid/Lipofectamine mix added to the monolayer before incubation at 37°C in a humidified 5% CO₂ atmosphere. After a further 4 hours, the cells were re-fed with EMEM-10. The cells were then typically used for investigation after 24 hours.

2.10.3 Amaxa nucleofection of siRNA oligonucleotides

Nucleofection of THP1 cells was performed using the Cell Line Nucleofector Kit V and a Nucleofector II machine (Amaxa Biosystems, Koeln, Germany). Cells were maintained at a density of 3-4 x 10⁵ cells/ml prior to nucleofection. The precise density of the cells was determined and 1 x 10⁶ cells per nucleofection centrifuged (90g, 5 min). The supernatant was discarded and the pellet re-suspended in 100 μ l of pre-warmed (to room temperature) Cell Line Nucleofector Solution V. 3 μ l of siRNA oligonucleotide was then added to the cell suspension and this mix transferred into a nucleofector cuvette. The cells were then nucleofected using program V-001 and the cell suspension immediately pipetted, using a fine Pasteur pipette, into pre-warmed RPMI-10 in a 6-well plate and the cells incubated at 37°C in a humidified 5% CO₂ atmosphere. The cells were then typically used for investigation 72 hours later.

2.11 siRNA oligonucleotides

All DNA oligonucleotides for siRNA experiments were obtained from Dharmacon Research (Lafayette, Colorado, USA) as 2'hydroxyl, annealed and desalted duplexes. If necessary, the siRNA was made to 20 μ M stocks using a dilution of the supplied 5 x buffer in RNase-free water. Scramble (scr) oligonucleotides with no known homology to mammalian sequences were used as controls. All siRNA oligonucleotides used are described below and both sense and antisense sequences given (5'-3').

2.11.1 scramble (scr)

GCGCGCUUUGUAGGAUUCG

CGAAUCCUACAAAGCGCGC

Scramble is commercially available and is also known as 'Scramble II duplex'.

2.11.2 hDaxx1

GGAGUUGGAUCUCUCAGAA

UUCUGAGAGAUCCAACUCC

sihDaxx1 has been previously described (Michaelson & Leder, 2003) and is also known as 'hDx2'. The duplex targets bp 678-698 of the human Daxx coding region (GenBank AF015956).

2.11.3 hDaxx2

CUACAGAUCUCCAAUGAAA

UUUCAUUGGAGAUCUGUAG

sihDaxx2 is commercially available (siGENOME D-004420-01) and targets bp 1613-1631 of the human Daxx coding region (GenBank NM_001350).

2.12.4 HP1 β

GCGCAAAGCUGAUUCUGAUA

UAUCAGAAUCAGCUUUGCGC

siHP1 β is available commercially (siGENOME D-009716-01) and targets bp 534-553 of the human CBX1 (HP1 β) coding region (GenBank NM_006807).

2.12.5 mDaxx

GGAGUUGGACCUGUCAGAG

CUCUGACAGGUCCAACUCC

simDaxx has been previously described (Michaelson & Leder, 2003) and is also known as 'mDx2'. The duplex targets bp 666-684 of the murine Daxx coding region (GenBank NM_007829).

2.12.6 Oct4

CAUCAAAGCUCUGCAGAAA

UUUCUGCAGAGCUUUGAUG

siOct4 is commercially available (siGENOME D-019591-05) and targets bp 468-486 of the human POU5F1 (Oct4) coding region (GenBank NM_002701).

2.12.7 PML

CCGAGGAGGCAGAGAGAGU

ACUCUCUCUGCCUCCUCGG

siPML is available commercially (siGENOME D-006547-01) and targets the human PML coding region (GenBank NM_002675).

2.12 Cloning and plasmid DNA preparation*2.12.1 Purification of PCR products*

Where it was necessary for PCR products to be cloned into plasmid vectors for the creation of standard plasmids for use in RTQ-PCR, the QIAquick Gel Extraction kit (Qiagen) was used as per the manufacturers guidelines.

PCR products, having been resolved on a 1-2% agarose gel, were excised from the gel using a scalpel and placed in a clean 1.5ml eppendorf. Having determined the mass of the gel fragment, three gel volumes of buffer QG (containing guanidine thiocyanate; 1mg ~ 1µl) were added to the gel and the sample heated at 50°C for 10 min with occasional vortexing. After dissolution of the gel, 1 gel volume of isopropanol was added to precipitate the DNA fragments and after mixing was added to a QIAquick spin column before centrifugation for 1 min at 16,000g. With the DNA bound to the column, the flow through was discarded and a further 0.5ml of buffer QG was added to the column and the sample spun once again. The column was then washed with the addition of 0.75ml of buffer PE and centrifugation again for 1 min at 16,000g. Any final residue liquid was then centrifuged out of the column before 50µl of buffer EB was added to the centre of the column and it left to stand for 1 min before centrifugation again at 16,000g for 1 min to elute the DNA.

2.12.2 Restriction enzyme digest

Restriction enzyme digests were used to prepare template plasmids for the cloning of PCR products and also for analysis of plasmid DNA preparations after cloning. Usually, digests

were carried out using 10 units of each enzyme in a final reaction mix of 20 μ l: 1 μ g DNA, 1 μ l enzyme, 2 μ l of specified enzyme buffer or 10x TAS buffer (330mM Tris-acetate, pH 7.9; 650mM potassium acetate; 100mM magnesium acetate; 5mM dithiothreitol; 40mM spermidine; 2.5mg/ml BSA) and 16 μ l dH₂O. The reaction mix was incubated at 37°C in a water bath for 1 hour. For analysis of digested plasmid DNA, the reaction was then resolved on a 1% agarose gel and the resultant digest pattern determined.

2.12.3 End filling of 5' protruding termini

In order to clone PCR products into a vector, restriction enzymes were used that created 5' protruding ends and the 3' recessed termini were then 'end filled' using DNA polymerase I large (Klenow) fragment (Promega) as follows: reaction mix was set up with 1 μ g DNA, 1 μ l Klenow enzyme, 5 μ l 10x buffer as supplied by the manufacturer, 5 μ l 25 μ M dNTP mix and dH₂O up to 50 μ l. This was then incubated at room temperature for 10 min before heat inactivation of the enzyme for 10 min at 75°C. The blunt end vector was then used for ligations.

2.12.4 Blunt end ligation

Ligations of blunt end vector plasmid and PCR products were carried out using T4 DNA ligase (Promega) and DNA concentrations such that a 1:1 molar ratio of vector to insert was maintained. Usually, reactions were set up as so: 5 μ l vector DNA, 5 μ l insert DNA, 2 μ l ligase 10x buffer, 2 μ l T4 DNA ligase and 6 μ l dH₂O. The mix was then incubated at 16°C overnight after which between 1-10 μ l of ligation reaction were used for transformation of bacteria.

2.12.5 Transformation

For transformations, 200 μ l of competent bacteria (*E. coli* DH5 α) were thawed on ice. Between 1-20 μ l of plasmid DNA was then added to the bacteria and the cells incubated on ice for a further 30 min. The bacteria were then heat shocked for 90 sec at 42°C before being placed back on ice for 1 min. 0.8ml of Luria-Bertani (LB) broth (10g tryptone, 5g yeast extract, 10g NaCl made up to 1 litre with dH₂O) was then added to the bacteria before incubation for a further 1 hour at 37°C. After this time, bacterial cells were pelleted at 16,000g for 30 sec and the supernatant decanted. The bacteria were then re-suspended in the residual volume and plated out on ampicillin (50 μ g/ml) agar plates (25ml of mix from:

5g tryptone, 2.5g yeast extract, 5g NaCl and 7.5g agar made up to 500ml with dH₂O) and incubated at 37°C overnight.

2.12.6 Small scale (Miniprep) plasmid preparation

A single colony of transformed bacteria was picked and grown up overnight in 2ml of LB broth supplemented with 50µg/ml ampicillin. After this time, 1.5ml of culture was pelleted at 16,000g for 1 min and then re-suspended in 100µl of resuspension buffer (25mM Tris-HCl; 50mM EDTA; 50mM sucrose; in dH₂O). The cells were then lysed using the alkaline SDS method whereby 200µl of lysis buffer (0.2M NaOH; 1% SDS (w/v); in dH₂O) was added to the sample and mixed. A further 150µl of 3M sodium acetate was then added to the reaction before it was again mixed and incubated on ice for 10 min. The cell debris was then pelleted (16,000g for 1 min) and the supernatant added to a fresh eppendorf tube before the addition of 1ml of ethanol (-20°C), the sample mixed and then incubated at -20°C for 20 min. Precipitated DNA was then pelleted by centrifugation (16,000g for 10 min), the supernatant removed and the pellet re-suspended in 100µl of 0.1M sodium acetate. The DNA was then re-precipitated by the addition of 200µl of ethanol (-20°C) and mixing. The samples were then centrifuged again (16,000g for 10 min), the supernatant removed and the final DNA pellet re-suspended in 50µl of dH₂O. To determine whether the plasmid DNA contained the required insert, restriction enzyme digestion was then carried out of 1µl of DNA as above (see section 2.12.2).

2.12.7 Large scale (Maxiprep) plasmid preparation

For the stock production of plasmids, purification was carried out using the Qiagen Maxi Kit (Qiagen) as per the manufacturer's guidelines. Usually, 500ml of LB broth supplemented with 50µg/ml ampicillin was used to culture transformed DH5α bacteria at 37°C in a shaking incubator (225rpm) overnight. The bacterial cells were then pelleted via centrifugation (3,800g for 15 min at 4°C; Sorvall SLA-1500 rotor) before re-suspension of the pellet in 10ml of buffer P1 (re-suspension buffer: 50mM Tris-HCl, pH 8.0; 10mM EDTA; 100µg/ml RNase A). The bacteria were then lysed by the addition of 10ml of buffer P2 (lysis buffer: 200mM NaOH; 1% SDS (w/v)) and the suspension mixed thoroughly before incubation at room temperature for 5 min. An additional 10ml of buffer P3 (neutralisation buffer: 3M potassium acetate, pH 5.5) was then added and the sample mixed again thoroughly before incubation on ice for 20 min. To clear the cell debris,

samples were centrifuged (17,200g for 30 min at 4°C; Sorvall SS-34 rotor) before the supernatant was placed into a fresh tube and centrifuged again (17,200g for 15 min at 4°C; Sorvall SS-34 rotor) to ensure removal of debris. Plasmid DNA was then harvested using Qiagen filter columns: 10ml of buffer QBT (equilibration buffer: 750mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton X-100 (v/v)) was added to a column to equilibrate the filter. The clean supernatant from the lysed bacteria containing the plasmid DNA was then added to the column and allowed to enter the resin by gravitational flow, leaving the DNA on the resin. The column was then washed twice with 30ml of buffer QC (wash buffer: 1M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v)) by gravity flow. The DNA was then eluted from the resin into a clean tube by the addition of 15ml of buffer QF (elution buffer: 1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% isopropanol (v/v)). The DNA was then precipitated by the addition of 10.5 ml of isopropanol to the solution, mixing and then centrifugation (17,200g for 30 min at 4°C; Sorvall SS-34 rotor). The supernatant was then decanted from the DNA pellet before washing of this with 5ml of 70% ethanol and centrifugation again (17,200g for 10 min at 4°C; Sorvall SS-34 rotor). Finally, the supernatant was decanted again and the DNA pellet allowed to air dry for 5-10 min before re-suspension in 0.5ml of dH₂O. The concentration of the plasmid DNA was then determined using a NanoDrop ND-1000 UV spectrophotometer and restriction digest carried out to confirm the structure of the plasmid DNA.

2.13 Plasmids

All plasmids were kindly prepared and provided by Joan Baillie and diluted to a concentration of 1µg/µl, unless otherwise stated.

2.13.1 *pcDNA3*

pcDNA3 is a commercially available plasmid (Invitrogen) and is designed for eukaryotic expression. It contains a multiple cloning site (MCS) into which coding regions can be inserted, placing them under the control of the minimal HCMV MIEP.

2.13.2 *pcDNA3-UL44prom*

pcDNA3-UL44prom constitutes the PCR product of the UL44 promoter, using the PCR primers in Table 2.3, cloned into the parent *pcDNA3* plasmid and was prepared by the author.

2.13.3 *pcDNA3-pp28prom*

pcDNA-pp28prom constitutes the PCR product of the *pp28* promoter, using the PCR primers in Table 2.3, cloned into the parent *pcDNA3* plasmid and was prepared by the author.

2.13.4 *pCMV-Oct4*

pCMV-Oct4 constitutes the *Oct4* coding region under the control of the minimal HCMV MIEP and has been previously described (Brehm *et al.*, 1997).

2.13.5 *pEScat*

pEScat is composed of the chloramphenicol acetyltransferase (*cat*) gene under the control of the full length MIEP (-2100 to +72) from HCMV strain AD169 and has been previously described (Shelbourn *et al.*, 1989b).

2.13.6 *pESluc*

pESluc constitutes the full length HCMV MIEP fused to the firefly luciferase gene in the *pGL3*-vector backbone.

2.13.7 *pFC-MEKK*

pFC-MEKK is composed of the Mitogen-Activated Protein (MAP) Kinase Kinase Kinase (MEKK) coding region 360-672 under the control of the HCMV MIEP. *pFC-MEKK* is commercially available as a PathDetect cis-Reporting system (Stratagene Europe, Amsterdam, The Netherlands).

2.13.8 *pGEM-T-Easy- γ globin*

pGEM-T-Easy- γ globin constitutes the PCR product of the γ -globin promoter, using the PCR primers in Table 2.3, cloned into the Promega *pGEM-T-Easy* plasmid and was supplied by Heather Coleman.

2.13.9 *pGEM-T-Easy-GAPDH*

pGEM-T-Easy-GAPDH constitutes the PCR product of the *GAPDH* promoter, using the PCR primers in Table 2.3, cloned into the Promega *pGEM-T-Easy* plasmid and was supplied by Heather Coleman.

2.13.10 *pISRE-luc*

pISRE-luc comprises an IFN- α/β -responsive element containing four tandem repeat sequences of the interferon-stimulated response element (ISRE) from the IFN-inducible gene fused to the firefly luciferase gene and is previously described (Poole *et al.*, 2006).

2.13.11 *pJATlac*

pJATlac is composed of the *Escherichia coli* β -galactosidase gene under the control of the constitutively active rat β -actin promoter and is previously described (Poole *et al.*, 2006).

2.13.12 *pNF- κ B-Luc*

pNF- κ B-Luc comprises a 5x repeat of the NF- κ B responsive element (TGGGGACTTTCCGC) fused to the firefly luciferase gene. *pNF- κ B-Luc* is commercially available as a PathDetect *cis*-Reporting system (Stratagene Europe, Amsterdam, The Netherlands).

2.14 RT-PCR analysis of RNA synthesis

2.14.1 Production of mRNA samples

RNA was isolated from cells using the RNazol B reagent (Biogenesis Ltd.) At the appropriate time point, 10^6 cells were lysed by direct addition of 0.5ml of reagent and repeated pipetting before aliquoting the sample. To this, 100 μ l of chloroform was added before vigorous shaking for 1 min and then incubation on ice for 5 min. The homogenate was then centrifuged at 16,000g for 15 min at 4°C. The upper aqueous layer was then removed and mixed with 0.5ml of isopropanol. After 10 min at room temperature, the sample was centrifuged at 16,000g for 5 min. The supernatant was removed and the RNA pellet was washed with 0.5ml of 75% ethanol/dH₂O and spun at 4000g for 5 min. The supernatant was again removed and the pellet air-dried for 5-10 min before re-suspension in 30 μ l of nuclease-free H₂O. RNA samples were then treated with DNase to preclude contamination. 1 μ l of DNase (Promega) and 1 μ l of DNase 10x buffer was added to 8 μ l of RNA in a 0.5ml eppendorf tube. This reaction mix was incubated at 37°C for 30 min before the addition of 1 μ l of DNase Stop Solution to terminate the reaction. The mix was then incubated again at 65°C for 10 min to inactivate the DNase and, if necessary, the RNA re-isolated by standard phenol/chloroform extraction and isopropanol precipitation as above.

2.14.2 Reverse transcription of mRNA

Reverse transcription was performed using the Promega Reverse Transcription kit. This uses the avian myeloblastosis virus reverse transcriptase (AMV RT) to synthesize single-stranded cDNA from total mRNA. The procedure was carried out as per the manufacturer's instructions using 5µl of harvested mRNA with incubation of the mix at 42°C for 20 min before an AMV RT denaturation incubation at 95°C for 5 min. Analysis of the produced cDNA was then carried out by PCR (see 2.8).

2.15 DNA extraction from cells

2.15.1 DNA extraction

DNA was isolated from cells using the sodium perchlorate (NaClO₄) method and dialysis of the resultant samples. Typically, 10⁶ cells from 1 well of a 6-well plate were scraped into the medium before centrifugation (4000g, 5 min). The supernatant was then discarded and the pellet was re-suspended in 0.6ml of 100mM NaCl / 5mM EDTA (pH 8.0). To this 125µl of 10% SDS was added and the cells lysed by pipetting. Further, 150µl of 5M NaClO₄ was then added to the sample and the solution agitated again by pipetting until the protein content had aggregated. DNA was then isolated by addition of 1ml of phenol:chloroform:isoamyl alcohol (25:24:1) and gentle mixing of the sample by inversion for 2 min before centrifugation (16000g, 15 min). The upper aqueous solution was then added to an equal volume of cold isopropanol (-20°C), shaken and then incubated at -20°C for at least 30 min. After this time, the sample was centrifuged (16000g, 30 min) and the supernatant discarded before washing of the DNA pellet with 0.5ml of 70% ethanol and further centrifugation (16000g, 10 min). The supernatant was again discarded and the pellet re-suspended in 100-200µl of TE buffer.

2.15.2 Dialysis of DNA samples

In order to eliminate excess salts that may interfere with PCR of the DNA samples, dialysis was used to further purify the isolated DNA. 5cm lengths of UltraPure 1/4 inch diameter dialysis tubing (Gibco GRL) were cut and washed in excess dH₂O. An end of each length was then clamped and a sample carefully added within before clamping of the opposite end and placing of all samples into an excess volume of TE buffer. The samples were then mixed slowly overnight at 4°C to allow dialysis to occur. After this time, one end of each tube was carefully unclamped and the dialysed samples added to fresh eppendorf tubes. If

necessary, the isopropanol precipitation method (see 2.14.1) was then again employed to isolate the DNA sample and aid re-suspension of all the samples in the appropriate volume. Analysis of the DNA samples was then carried out by PCR (see 2.8).

2.16 Luciferase assay analysis

2.16.1 Luciferase assay

Cells were typically seeded on 6-well plates and transfected (see 2.10.2) with the appropriate luciferase constructs and controls 24 hours before analysis. After this time, the cells were washed in 2ml of PBS before addition of 200 μ l of luciferase buffer A (25mM Tris phosphate, pH 7.8; 8mM MgCl₂; 1mM DTT; 1mM EDTA; 1% Triton X-100 (v/v); in dH₂O) for 2 min to lyse the cells. Then 200 μ l of luciferase buffer B (25mM Tris phosphate, pH 7.8; 8mM MgCl₂; 1mM DTT; 1mM EDTA; 1% Triton X-100 (v/v); 0.8mM ATP; 2% BSA (w/v); 30% glycerol (v/v); in dH₂O) was added and the solutions mixed by gentle swirling before incubation of the plates at -20°C overnight to aid lysis of the cells. Plates were then thawed and lysed cells suspended by gentle pipetting before transfer to an eppendorf tube and centrifugation (4000g, 1 min) to remove cellular debris. The supernatant was then transferred to a luminometer tube (75 x 12mm, Sarstedt) and the samples analysed using an AutoLumat LB953 reader (Berthold) with automatic injection of 100 μ l of 0.6mM luciferin to each sample. Background levels were detected using untransfected cell samples for analysis.

2.16.2 β -galactosidase assay

To normalise luciferase assay results to transfection efficiencies, cells were co-transfected with a β -galactosidase construct (pJATlac, see 2.12.9) and the following analysis carried out. After luciferase analysis, 1ml of LacZ buffer/ONPG (60mM Na₂HPO₄·7H₂O; 40mM NaH₂PO₄·H₂O; 10mM KCl; 0.3% β -mercaptoethanol (v/v); 1mM MgSO₄·7H₂O) was added to each sample and the tubes incubated at 30°C. When the samples had turned yellow, the reaction was terminated by addition of 0.5ml of 1M Na₂CO₃ and the optical density at 420nm (OD₄₂₀) determined by transfer to a 1ml cuvette (Sarstedt) and analysis using a BioSpec-1601E spectrometer (Shimadzu) for each sample against a control of mock transfected cells. The luciferase assay results were then normalised using the β -galactosidase results.

3. Results

The MIEP of HCMV is chromatinised throughout productive infection

3.1 Introduction

It is now well established that the transcriptional regulation of gene expression in eukaryotic organisms is mediated by the modulation or remodelling of chromatin structure associated with promoters and coding sequences of their genes. Investigation over the last thirty years has led to a substantial increase in our knowledge of the modifications made to chromatin structure and the enzymes which carry this out. Indeed, this has led to a consensus that particular modifications at certain positions along gene promoters and coding regions are in fact hallmarks for repression or activation of gene expression and that they can be used to determine the transcriptional activity of the gene (see Fig. 1.11) (reviewed in: Kouzarides, 2007a).

In addition, it is becoming increasingly clear that the differentiation-dependent control of HCMV gene expression is regulated by the chromatin status of the viral MIEP. Studies using conditionally permissive cell models, such as T2 cells, cultured monocytes and CD34⁺ cells, have provided a wealth of information regarding the chromatin status of the MIEP. In the undifferentiated, non-permissive exogenously infected cells, the MIEP is associated with HP1 β , a marker of transcriptional repression, whereas in the differentiated, permissive, exogenously infected cells the MIEP becomes associated instead with high levels of acetylated histone-H4 (Murphy *et al.*, 2002; Reeves *et al.*, 2005a). It has also been shown that in the THP1 cell line, which is differentiation-dependently permissive for HCMV IE gene expression (Sinclair *et al.*, 1992), the viral MIEP is associated with acetylated histone-H3 at lysine 9 after differentiation to macrophage, with the absence of this residue in undifferentiated, non-permissive cells (Ioudinkova *et al.*, 2006). These observations in experimentally infected cell models of latency have also been recapitulated in natural latency in CD34⁺ cells where the same repression of IE gene expression through repression of the viral MIEP occurs (Reeves *et al.*, 2005b). In addition, the maturation of these cells to fully mature DCs results in reactivation of endogenous latent virus concomitant with the association of acetylated histone H4 with the viral MIEP (Reeves *et al.*, 2005b).

The gene expression of a number of other viruses has been shown to be regulated through chromatinisation of viral promoters and remodelling of chromatin. Examples include other herpesviruses, such as EBV, KSHV and HSV-1, where the control of latency and reactivation also appears to be tightly regulated by the chromatin status of certain promoters. Furthermore, the control of gene expression of different classes of viral genes throughout a productive, lytic infection of fully permissive cells with HSV-1 has also been shown to be mediated by chromatinisation and remodelling of promoters (Herrera & Triezenberg, 2004; Kent *et al.*, 2004; Coleman *et al.*, 2008). Similarly, in the case of MCMV, productive infection appears to be regulated by the chromatinisation of the viral MIEP: the promoter appears to be repressed to some degree during a lytic infection as the use of HDAC inhibitors causes increased IE gene expression and infectious virus progeny output (Tang & Maul, 2003). Furthermore, the impairment in growth of an IE72-deficient mutant HCMV can be rescued with the use of HDAC inhibitors (Nevels *et al.*, 2004b). This repression has been hypothesised to be mediated by chromatin remodelling enzymes present at ND10 where the viral genome is deposited upon infection. Therefore, it was first decided to investigate whether the MIEP of HCMV is chromatinised throughout a productive infection of fully permissive human fibroblasts and whether modulation of the chromatin structure occurs throughout infection.

3.2 Results

3.2.1 *The MIEP is associated with both acetylated and methylated histones throughout productive infection*

Human fibroblasts, fully permissive for HCMV lytic infection, were first incubated for 48 hours with serum-free medium to synchronise the cells in the G₀/G₁ phase of the cell cycle prior to infection, such that the initiation of gene expression from infecting genomes would also be synchronised. After this, cells were infected in serum-containing medium at a low multiplicity of infection (MOI 0.1). At the appropriate timepoint (3, 24 or 96 hours post-infection) the protein/DNA chromatin complexes within the cell populations were fixed with formaldehyde (1%) and ChIP assays carried out using either control serum (Con), an anti-acetylated histone-H4 antibody (H4ac), an anti-di-methyl lysine 9 histone-H3 (H3K9me2) antibody or an anti-HP1 β (HP1 β) antibody. Immunoprecipitated DNA fragments were then amplified using viral MIEP specific primers, complementary to positions -272 to +13 of the transcriptional start site (Murphy *et al.*, 2002), with 10% of the

total chromatin fraction being used as a relative input for each timepoint, and the products separated on a 2% agarose gel. To aid specificity of the analysis, Southern blotting was then carried out using a probe internal to the initial PCR primers and the resultant autoradiograph bands from each sample are displayed in Fig. 3.1.

Throughout the timecourse of infection, all amplification of input DNA provided visible levels after Southern blotting (Fig. 3.1, lane 1) and immunoprecipitation with control serum showed little to no precipitated DNA levels (Fig. 3.1, lane 2). Upon analysis of the antibody immunoprecipitated samples, the viral MIEP appeared to be associated with histones and histone associated proteins across all timepoints. However, at this stage, to better compare the PCR data from the ChIP samples, Image J analysis was used to quantify the intensity of the bands from the autoradiograph and presented in a graphical form seen in Fig. 3.2. This representation also contains data from triplicate PCRs and the level of immunoprecipitated DNA is quantified relative to the input DNA for that timepoint, taken as 1.0. This more quantitative data was then used to analyse the results.

At 3 hours post-infection with HCMV, the viral MIEP was associated with acetylated histone-H4 (Fig 3.2a, lane 3), only minimally and, intriguingly, the incoming genomes also appeared to be associated with both H3K9me2 and HP1 β (Fig. 3.2a, lane 4 & 5 respectively). Since both H3K9me2 and HP1 β modifications are markers for silenced chromatin (Kouzarides, 2007a), this would suggest that a significant proportion of MIEPs are associated with transcriptionally inactive chromatin immediately upon infection.

At 24 hours post-infection, the proportion of viral MIEPs associated with acetylated histones substantially increased (Fig. 3.2b, lane 3) by as much as six-fold, which is consistent with the known increase in IE gene expression prior to the onset of early and late gene expression. In addition, the levels of both H3K9me2 and HP1 β associated with the viral MIEP decreased substantially (Fig. 3.2b, lanes 4 & 5 respectively). Again, this is consistent with an active transcriptional chromatin conformation of the promoter at this time.

At late times (96 hours post-infection), the largest change in the chromatin structure of the MIEP was a predominant re-association of HP1 β (Fig. 3.2c, lane 5) as well as a decrease in

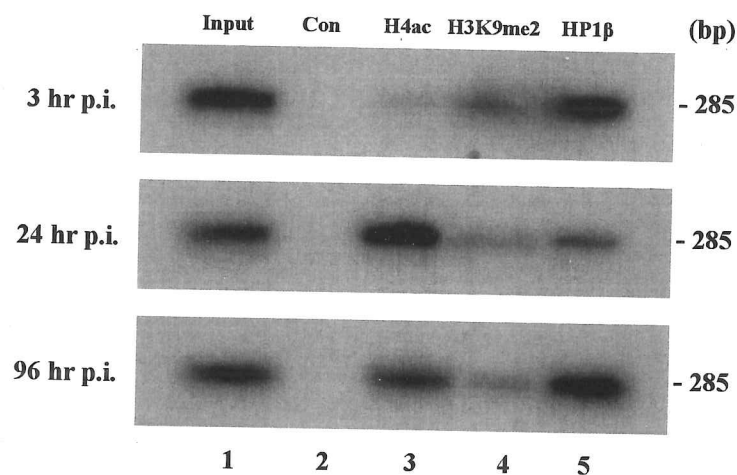


Figure 3.1 The MIEP of HCMV is chromatinised throughout a productive lytic infection. ChIP assays were performed on serum-starved human fibroblasts infected with HCMV Toledo at 3, 24 and 96 hours post-infection (MOI 0.1). Analysis was carried out with either control serum (Con, lane 2), an anti-acetylated histone-H4 antibody (H4ac, lane 3), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 4) or an anti-HP1 β antibody (HP1 β , lane 5). Inputs (10% of total chromatin) are shown (Input, lane 1). Isolated DNA was amplified using an MIEP-specific PCR and products run on a 2% agarose gel before Southern blot analysis.

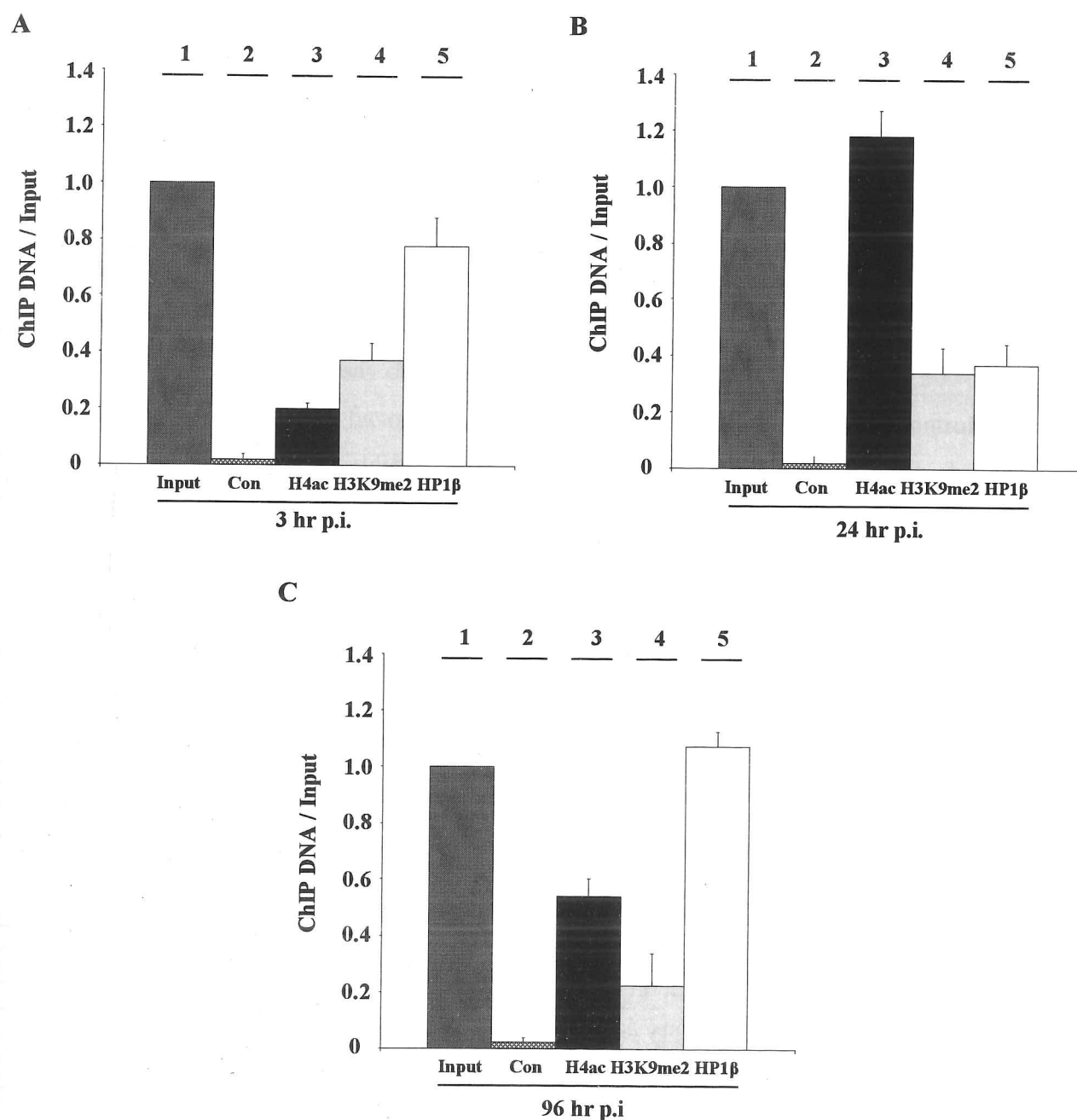


Figure 3.2 The MIEP of HCMV is chromatinised throughout a productive lytic infection – graphical representation. ChIP assays were performed on serum-starved human fibroblasts at 3, 24 and 96 (A-C) hours post-infection (MOI 0.1). Analysis was carried out with either control serum (Con), an anti-acetylated histone-H4 antibody (H4ac), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2) or an anti-HP1β antibody (HP1β). Inputs are shown (Input). Isolated DNA was amplified using an MIEP-specific PCR and products run on a 2% agarose gel before Southern blot analysis. Image J analysis was then used to quantify autoradiograph bands. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

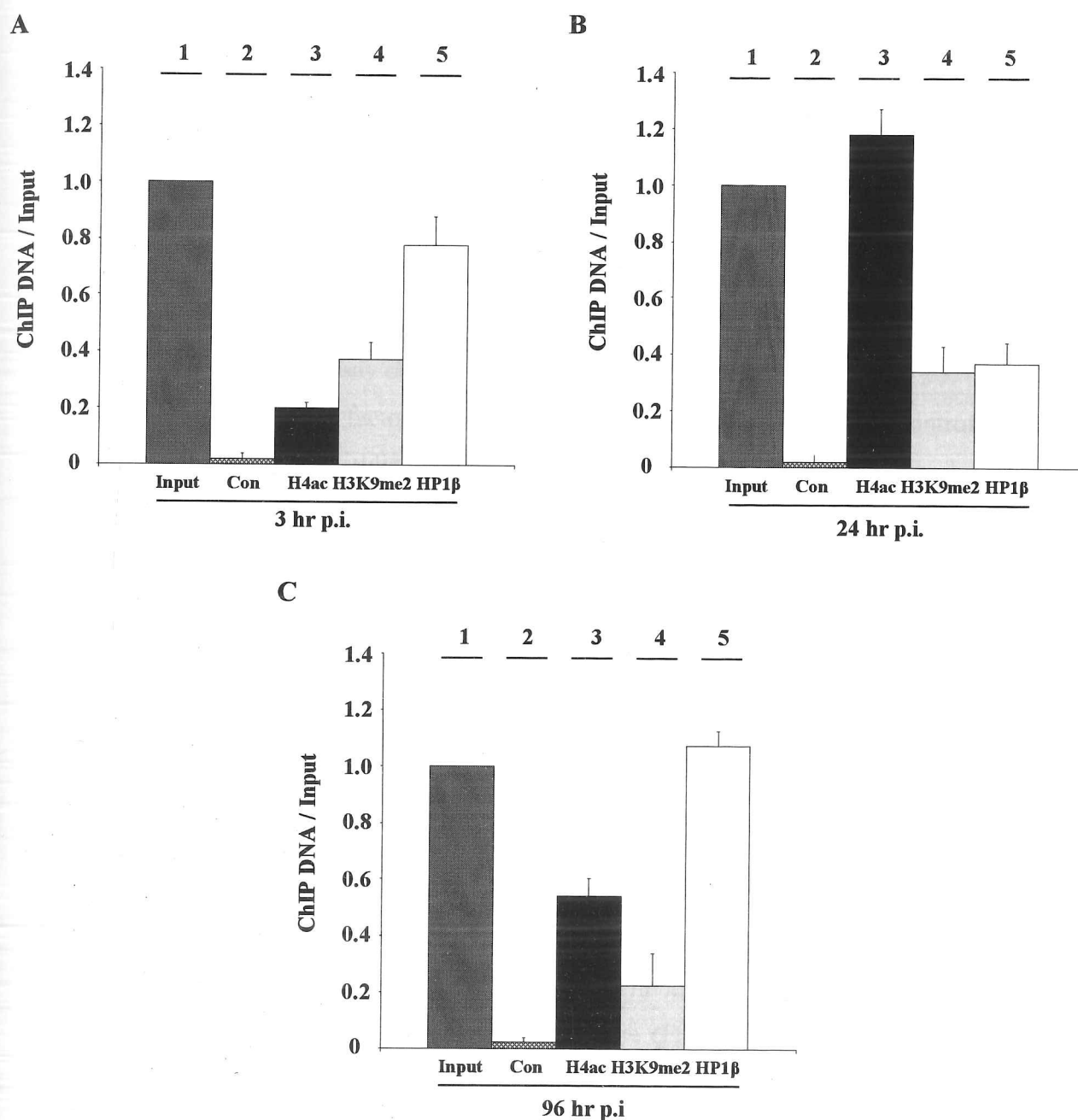


Figure 3.2 The MIEP of HCMV is chromatinised throughout a productive lytic infection – graphical representation. ChIP assays were performed on serum-starved human fibroblasts at 3, 24 and 96 (A-C) hours post-infection (MOI 0.1). Analysis was carried out with either control serum (Con), an anti-acetylated histone-H4 antibody (H4ac), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2) or an anti-HP1β antibody (HP1β). Inputs are shown (Input). Isolated DNA was amplified using an MIEP-specific PCR and products run on a 2% agarose gel before Southern blot analysis. Image J analysis was then used to quantify autoradiograph bands. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

the level of acetylated histone-H4 association (Fig. 3.2c, lane 3). This is consistent with the known negative auto-regulation of IE gene expression at late times of infection through the repression of the viral MIEP by IE86 (Meier & Stinski, 1996; Reeves *et al.*, 2006). It is unclear why H3K9me2 association with the MIEP appears to decrease at this time (Fig. 3.2c, lane 4). However, this may be due to the increase in HP1 β association which could mask the immunoprecipitation of di- and tri-methyl residues on the histone tail (Bannister *et al.*, 2005).

To ensure consistent immunoprecipitation of specific histone or histone associated proteins across each timepoint and throughout the full timecourse of infection, it was necessary to carry out a control PCR assay on each sample. Previous studies (Nevels *et al.*, 2004b) have used c-Fos, a transcription factor constitutively expressed in fibroblasts, as a control for the immunoprecipitation of acetylated histone-H4 during infection with HCMV which showed little change in the chromatin associated with the c-Fos promoter. Therefore, this control was also employed here. For all samples, a PCR was carried out specifically for the c-Fos promoter. The resultant amplified products were resolved on a 2% agarose gel and viewed using ethidium bromide staining and ultraviolet light (Fig. 3.3). The bands present were then analysed using Image J analysis, as described above, and the quantitative representation is provided in Fig 3.4.

UV analysis of the PCR products showed that the c-Fos promoter was predominantly associated with acetylated histone-H4 throughout infection as expected (Fig. 3.3, lane 3). However, levels of both H3K9me2 and HP1 β , although less than acetylated histone-H4, were apparent (Fig. 3.3, lanes 4 & 5, respectively). The level of precipitation of acetylated histone-H4 varied slightly throughout the timecourse (Fig. 3.4a, lanes 1-3) although not significantly when compared quantitatively. Indeed, this was also the case for the two markers of repression (Fig. 3.3b & 3.3c). Hence, the immunoprecipitation of chromatin had occurred consistently throughout the analysis.

Therefore, although the MIEP of HCMV is associated with both acetylated and methylated histones throughout infection, the overall state of chromatinisation of the promoter with respect to the known markers of transcriptional activation or repression is consistent with the levels of major IE transcription at these times. However, the MIEP does appear to be

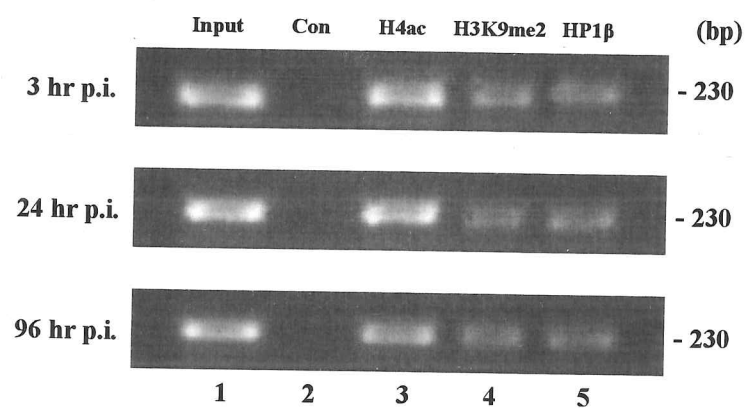


Figure 3.3 PCR of the c-Fos promoter shows consistent immunoprecipitation of chromatin. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 24 and 96 hours post-infection. Analysis was carried out with either control serum (Con, lane 2), an anti-acetylated histone-H4 antibody (H4ac, lane 3), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 4) or an anti-HP1 β antibody (HP1 β , lane 5). Inputs (10% of total chromatin) are shown (Input, lane 1). Isolated DNA was amplified using an c-Fos-specific PCR and products run on a 2% agarose gel and visualised using UV light.

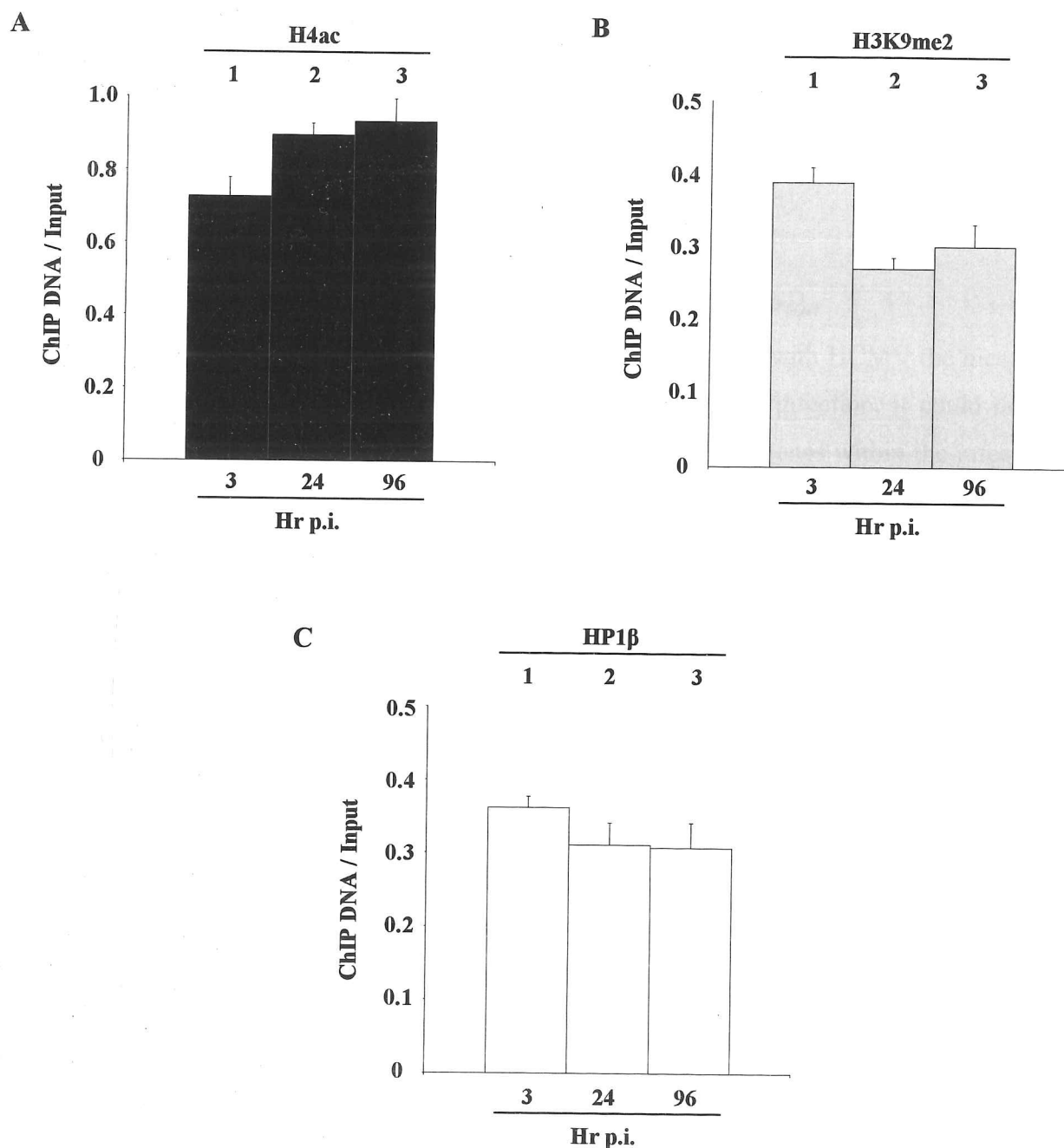


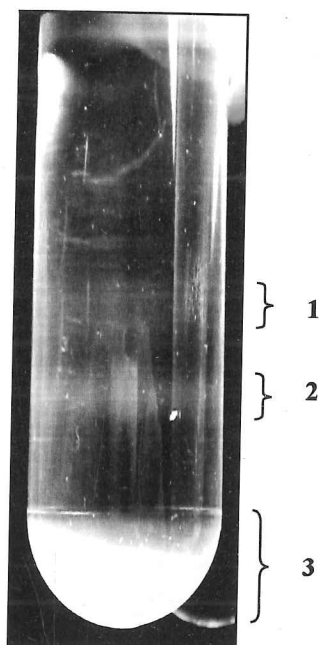
Figure 3.4 The chromatinisation of the c-Fos promoter does not change throughout viral lytic infection. ChIP assays were performed on HCMV Toledo infected (MOI 0.1), serum-starved human fibroblasts at 3, 24 and 96 hours post-infection. Analysis was carried out with either (A) an anti-acetylated histone-H4 antibody, (B) an anti-dimethyl lysine 9 histone-H3 antibody or (C) an anti-HP1β antibody. Isolated DNA was amplified using a c-Fos-specific PCR and products run out on a 2% agarose gel stained with ethidium bromide. Image J analysis was then used to quantify bands and represented as above. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

associated with a level of repressive chromatin immediately upon infection and from this analysis it cannot be established whether this association of repressive chromatin occurs upon infection and entry of naked viral DNA to the nucleus or whether the viral genome is packaged into the virion core with histone proteins after replication and thus enters in this form.

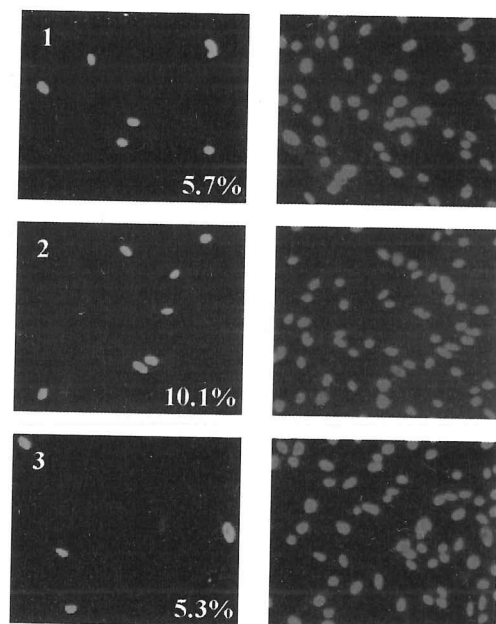
3.2.2 *The HCMV genome is not associated with histones in the virion*

Although the ChIP analysis had determined that upon infection with HCMV the incoming viral genomes were chromatinised and remodelled throughout infection, it could not be precluded that the viral genome was already associated with histones within the infectious virion. Therefore, to determine if this was so, infectious viral particles were first isolated from crude clarified virus preparations via density centrifugation. Initially, a gradient of 5-15% Ficoll was used as a medium to separate the particles present in the concentrated, crude HCMV stock (Fig. 3.5a) as has been performed previously to separate HSV-1 particles from non-infectious by-products (Szilagyi & Cunningham, 1991). This separation of crude HCMV infected fibroblasts supernatants produced three bands as expected: non-infectious enveloped particles (NIEPs), infectious virions and dense bodies (DBs). After harvest of each band, their infectivity was tested on fibroblasts (Fig. 3.5b). After fixation and staining of infected cells for IE72/86 expression at 24 hours post-infection, it was found that although band 2 was associated with the highest level of infectious virus (47.9% of total; Fig. 3.5c), all bands showed infectivity over 25%. Purified bands of NIEPs and DBs, due to the absence of packaged genome, should not show any infectivity (Irmiere & Gibson, 1983), suggesting that all bands were contaminated with infectious virions. Therefore, a further method was developed with the aim of isolating a purer preparation of infectious virions. This protocol was based on that used by the Compton group to purify HCMV infectious particles using a 20-70% sorbitol gradient (Reeves & Woodhall, personal communication) (Fig. 3.6a). With this method, the separation now produced five bands. These bands were, again, harvested and tested for infectious virus on fibroblasts with subsequent staining for IE72/86 expression after 24 hours as before (Fig. 3.6b). This showed that only bands 3 and 4 contained high levels of infectious virions, with bands 1, 2 and 5 showing low levels of infection. Furthermore, infection with band 3 demonstrated the highest level of infected cells (58.8%) which corresponded to 50.3% of the total infectious virions isolated from the gradient (Fig. 3.6c). Indeed, the level of infectious

A



B



C

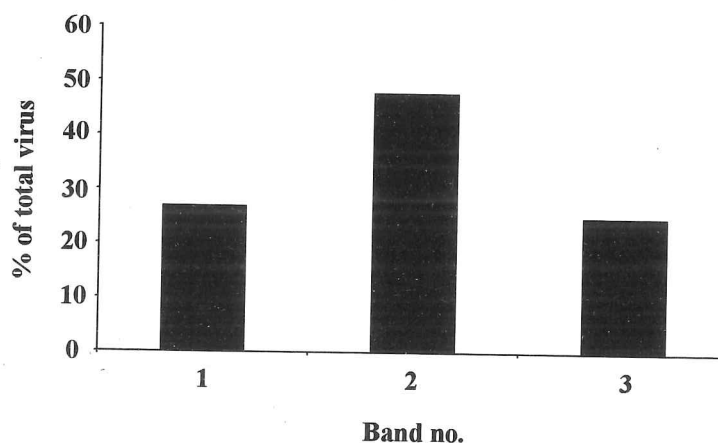


Figure 3.5 Ficoll gradient purification of HCMV Toledo particles. (A) A clarified virus preparation of HCMV Toledo was separated by high speed centrifugation (19,000g) through a Ficoll gradient (5-15%). (B) The visible bands were then harvested, pelleted and used to infect human fibroblasts. At 24 hours post-infection cells were fixed and stained for IE72/86 (green). Hoechst 33342 staining (blue) was used throughout to identify cell nuclei. The percentage of infected cells is stated in the bottom right corner of each view. (C) The percentage of infectious virus in each band was subsequently calculated from the total harvested from all the bands.

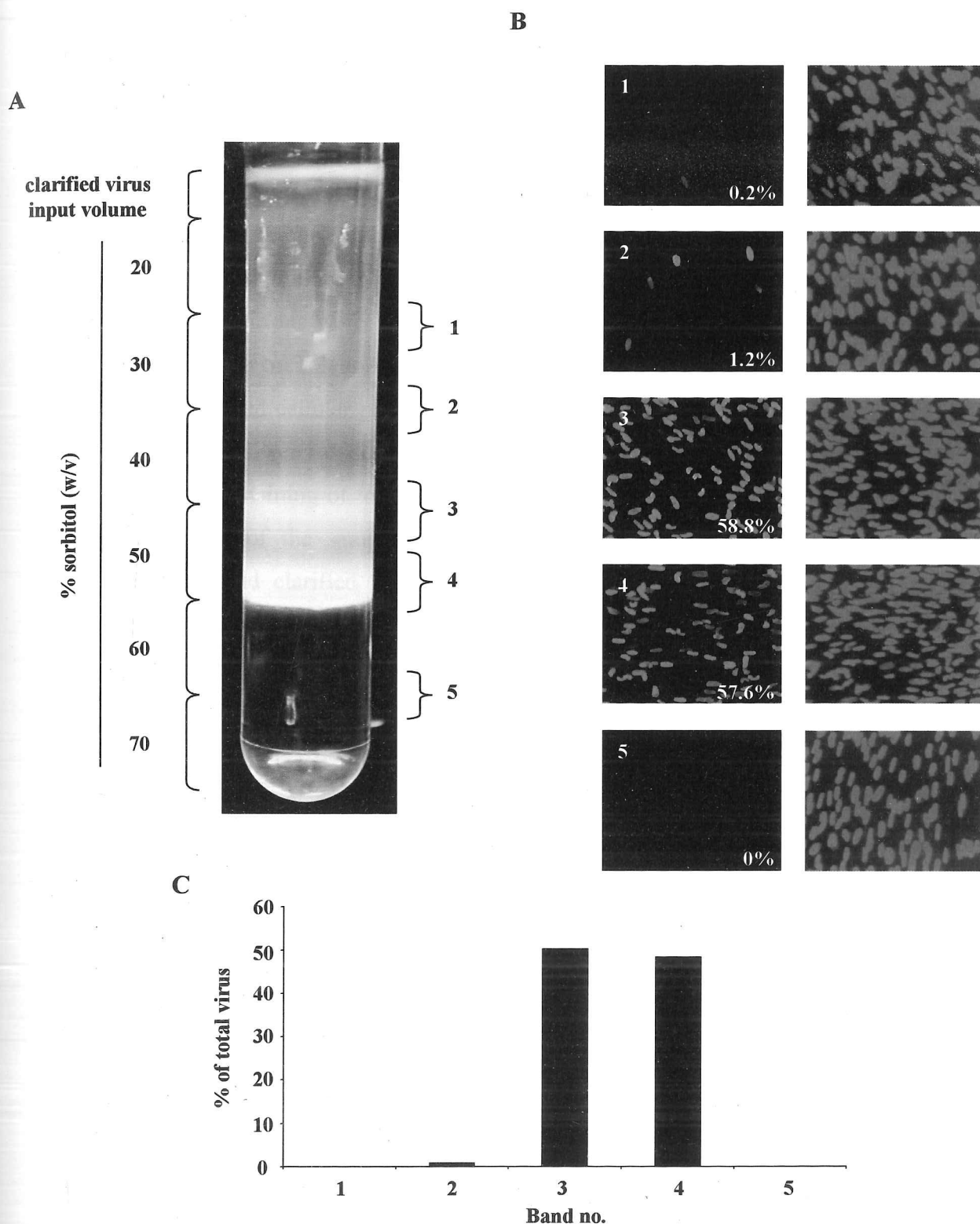


Figure 3.6 Sorbitol gradient purification of HCMV Toledo particles. (A) A clarified virus preparation of HCMV Toledo was separated by high speed centrifugation (49,500g) through a Sorbitol gradient (20-70%). (B) The visible bands were then harvested, pelleted and used to infect human fibroblasts. At 24 hours post-infection cells were fixed and stained for IE72/86 (red). Hoechst 33342 staining (blue) was used throughout to identify cell nuclei. (C) The percentage of infectious virus in each band was subsequently calculated from the total harvested from all the bands.

virus isolated from the sorbitol gradient was approximately six-fold greater than that achieved with a Ficoll gradient. As such, band 3 of the sorbitol purified virions was used for all subsequent experimentation requiring purified virus.

In order to determine whether the viral genome was packaged within the virion already associated with histones, the protein constituents of purified infectious virions were analysed by Western blotting (Fig. 3.7). Three separate preparations of purified infectious HCMV Toledo virions were analysed (Fig. 3.7, lanes 1-3) in addition to crude clarified virus preparations of Toledo, TB40/E and Towne-GFP (Fig. 3.7, lanes 4, 6 & 7) and an uninfected clarified cellular pellet (Fig. 3.7, lane 5). 1×10^5 plaque forming units (PFU) of virus preparations were loaded and an equivalent volume of uninfected clarified material. Coomassie blue staining of total protein loading was used as a loading control due to the differing nature of the samples and showed approximate equal loading between the purified virus and clarified virus samples (Fig. 3.7, lanes 1-7). Staining for pp28, a tegument protein present in the infectious virion (Spaete *et al.*, 1994), confirmed that all virus preparations, both clarified and purified, had virions present (Fig. 3.7, lane 1-4, 6 & 7) and that the control uninfected clarified pellet did not (Fig. 3.7, lane 5). However, upon staining for histone-H3 it was found that although all clarified pellets, both infected and uninfected, were associated with histone-H3 (Fig. 3.7, lanes 4-7), no histone-H3 could be detected in protein isolated from purified HCMV Toledo preparations (Fig. 3.7, lanes 1-3). Indeed, this was also observed when staining for the modified histone form, H3K9me2, whereby no histone was found associated with the purified virus preparations (Fig. 3.7). This finding is not entirely unexpected since it is consistent with findings from other groups using amino acid sequence analysis and mass spectrometry where no histone proteins have been found with purified virions (Baldick & Shenk, 1996; Varnum *et al.*, 2004). Consequently, histones are not associated with the viral genome within the virion.

3.2.3 TSA treatment of permissive fibroblasts increases the level of IE gene expression

ChIP assays of productively infected, permissive human fibroblasts had shown that the viral MIEP appears to be associated with markers of repressive chromatin, such as methylated histones and HP1 β , at very IE times (Fig. 3.1). The establishment of any chromatin structure around the MIEP would be dependent upon various chromatin remodelling enzymes, including histone deacetylases (HDACs). Therefore, to test whether

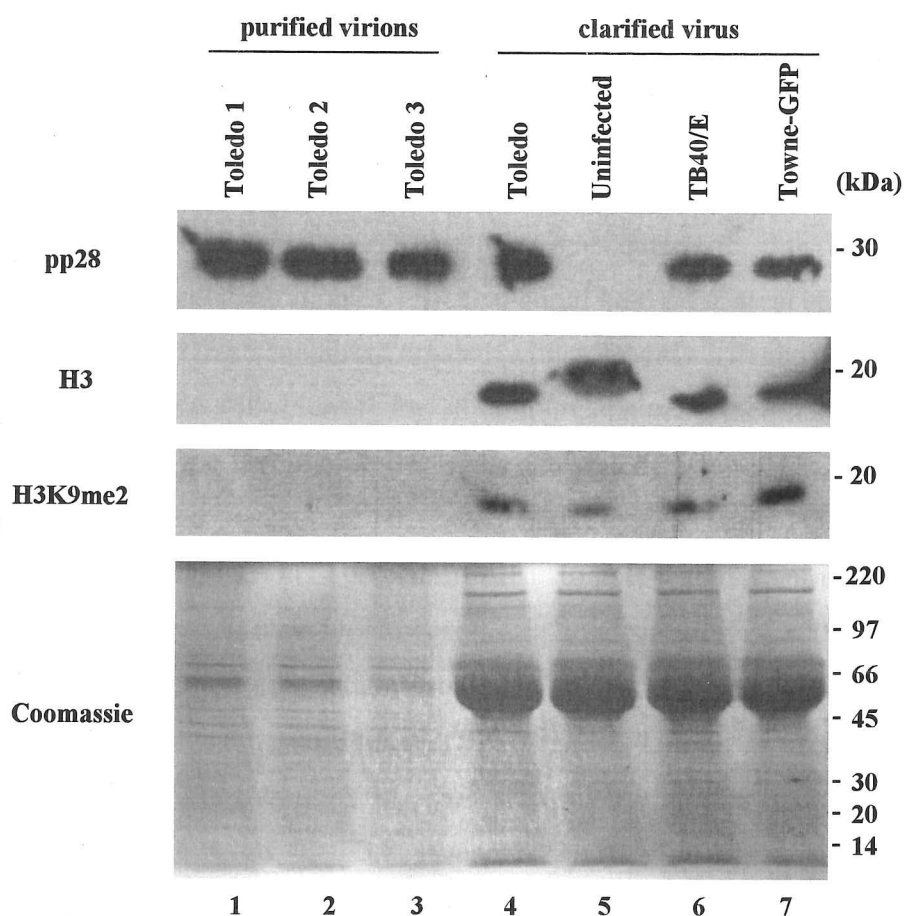


Figure 3.7 Purified infectious HCMV virions are not associated with histones. Purified virions from three HCMV strain Toledo infections (lanes 1-3), clarified virus preparations of strains Toledo (lane 4), TB40/E (lane 6) and Towne-GFP (lane 7) and uninfected clarified cell debris (lane 5) were re-suspended in Laemmli's buffer and run on a 10% polyacrylamide gel to separate the protein content. Western blotting was then used to analyse viral late (pp28), histone-H3 (H3) and dimethyl-lysine 9 histone-H3 (H3K9me2) levels. Coomassie blue staining was used as a loading control.

this repressive chromatin structure of the MIEP at this time of infection was actually correlated with repression of viral gene expression, the use of a HDAC inhibitor Trichostatin-A (TSA) was employed. The addition of TSA to normally non-permissive, undifferentiated T2 cells has previously been shown to render them permissive for HCMV infection (Meier, 2001; Murphy *et al.*, 2002) and treatment of normally permissive mouse embryonal fibroblasts (MEFs) before infection with MCMV has also been shown to lead to increases in viral gene expression and production of infectious virus (Tang & Maul, 2003). However, prolonged exposure to TSA can also be cytotoxic, thus it was necessary to establish the lowest concentration of TSA that could be used to treat cells. Fibroblasts were either mock treated (fresh medium), treated with a DMSO solvent control or a range of concentrations of TSA (50-450nM) for 16 hours. Cells were then harvested and analysed by Western blot for the levels of endogenous acetylated histone-H4 under each treatment (Fig. 3.8). DMSO and 50-250nM TSA treatment (Fig. 3.8, lanes 2-7) caused little or no increase in global endogenous acetylation of histone-H4 above normal levels seen with mock treatment (Fig. 3.8, lane 1). However, at a concentration of 300nM, TSA causes an increase in the level of acetylated histone-H4 (Fig. 3.8, lane 8) and this increase is maximised before plateauing at 330nM TSA (Fig. 3.8, lane 9; as used in: Murphy *et al.*, 2002) with higher concentrations of TSA showing no further increase in endogenous acetylated histone-H4 levels (Fig. 3.8, lanes 10-12). As global histone-H4 acetylation had been observed, and no ill effects to the cells had been found with GAPDH loading levels remaining consistent across the range of TSA concentrations, all further TSA treatment of cells was conducted with TSA at a concentration of 330nM.

To determine the effects of TSA on viral IE gene expression, the steady state levels of viral RNA were first assessed. Fibroblasts were treated with fresh medium (mock), DMSO or TSA for 16 hours before the cells were infected with HCMV Toledo (MOI 0.5). After 24 hours, total RNA was harvested and reverse transcription followed by PCR (RT-PCR) was performed to determine relative levels of GAPDH, viral IE genes and the abundant viral $\beta 2.7$ early RNA (McSharry *et al.*, 2003). DMSO treated cells (Fig. 3.9, lane 2) showed normal levels of IE RNA after 24 hours in comparison to mock treated cells (Fig. 3.9, lane 1) and both showed similar levels of the $\beta 2.7$ transcript present within infected cells. However, TSA treatment prior to infection showed a clear increase in the abundance of IE RNA levels (Fig. 3.9, lane 3) when compared to the control samples. Concomitant with

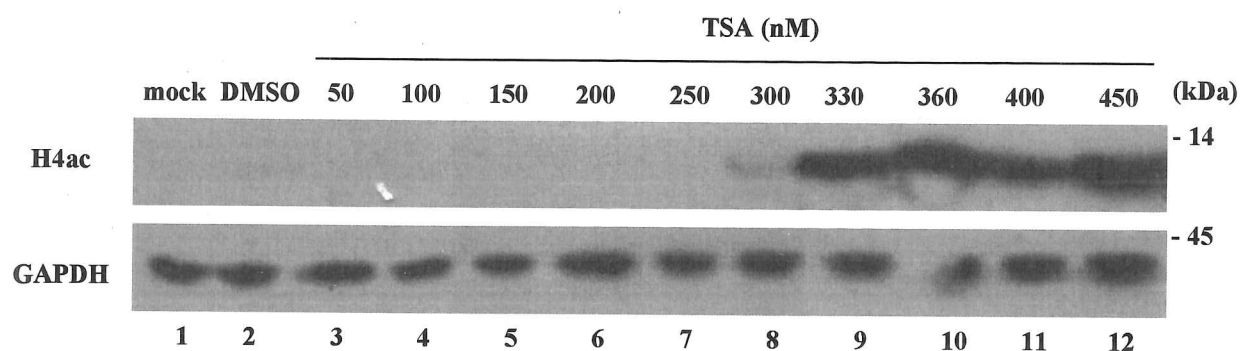


Figure 3.8 Trichostatin A (TSA) causes global acetylation of histone-H4. Human fibroblasts were either mock treated (fresh media; lane 1), treated with DMSO media (lane 2) or with TSA media at a range of concentrations (50-450nM; lanes 3-12) for 16 hours. Cells were then harvested and protein samples run on a 10% polyacrylamide gel before Western blot analysis for acetylated histone-H4 (H4ac) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control.

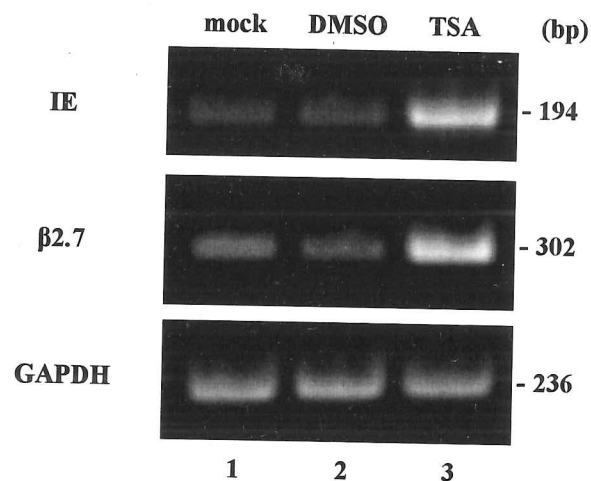


Figure 3.9 TSA treatment causes an increase in viral IE and early RNA production. Human fibroblasts were treated with either fresh (mock), DMSO or TSA media. After 16 hours, cells were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and extraction of total RNA. After reverse transcription, specific PCR was carried out for viral IE genes, viral early transcript $\beta 2.7$ and GAPDH as a loading control.

this, an increase in $\beta 2.7$ transcript levels was also apparent in the TSA treated infected fibroblasts (Fig. 3.9, lane 3).

Having found that TSA treatment prior to HCMV infection did indeed lead to an increase in IE transcription levels, it was next decided to assess the effects of TSA on viral protein production. A parallel analysis was undertaken whereby fibroblasts were again treated with either fresh medium (mock), DMSO or TSA for 16 hours before the cells were infected with HCMV Toledo (MOI 0.5). After 24 hours, protein samples were taken and Western blot analysis for IE gene expression levels carried out (Fig. 3.10a). Consistent with the RNA analysis, DMSO treated cells (Fig. 3.10a, lane 2) showed a similar level of viral IE protein production as mock treated cells (Fig. 3.10a, lane 1). However, as with RNA levels, treatment of fibroblasts with TSA led to an overt increase in IE72 and IE86 protein levels (Fig. 3.10a, lane 3) compared with levels seen in control samples.

In order to further examine the effect of TSA on viral IE gene expression, the permissive U373 cell line was employed and similar analysis carried out to ensure that the effects seen in human fibroblasts were not cell-specific. U373 cells were treated as above and after 16 hours infected with HCMV Toledo (MOI 0.5). After a further 24 hours, protein samples were taken and Western blot analysis for IE gene expression levels again carried out (Fig. 3.10b). The control mock and DMSO treated cells again showed similar levels of IE72 and IE86 expression (Fig. 3.10b, lane 1 & 2). However, again TSA treatment of U373 cells prior to infection with HCMV led to an obvious increase in IE72 and IE86 protein production (Fig. 3.10b, lane 3) when compared to the control samples. Therefore, TSA treatment had a similar effect on U373 cells as it had on human fibroblasts.

Since analysis of both viral RNA and protein levels had shown that DMSO treatment had no profound differential effect than the mock control, a sole DMSO control was used when treating cells with TSA from here on in.

3.2.4 TSA treatment increases the level of permissiveness of lytic infection at low multiplicities of infection and levels of IE gene expression within an infected cell

Inhibition of HDACs by TSA had led to an increase in both IE transcription levels and abundance of IE protein by 24 hours post-infection. However, RT-PCR and Western blot

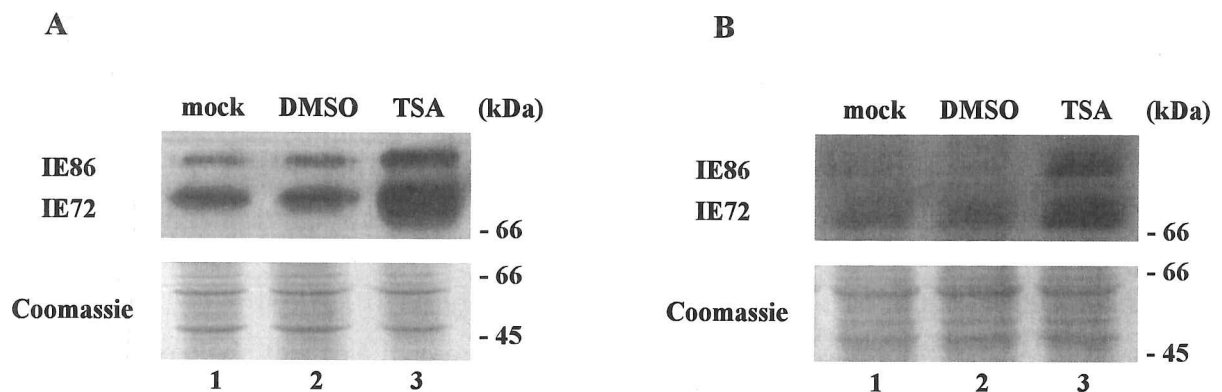


Figure 3.10 TSA treatment causes increases in viral IE protein production. (A) Human fibroblasts were treated with either fresh (mock; lane 1), DMSO (lane 2) or TSA (lane 3) media. After 16 hours, cells were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and analysis of protein content by Western blot for IE protein expression. Coomassie stained gels are presented as loading controls. (B) U373 cells were treated and analysed as above.

analysis does not distinguish between an increase in viral IE gene expression in individual cells or an increase in the number of cells productively infected. Therefore, it was necessary to determine whether TSA simply increased levels of IE gene expression in cells already producing IE RNA or whether it increased the frequency of cells initiating IE gene expression. To this end, fibroblasts were treated with either DMSO or TSA media for 16 hours before infection with HCMV Toledo at a low MOI (0.05). After 24 hours, cells were then fixed and stained for IE72/86 expression with counter-staining of nuclei through Hoechst 33342 and viewed under a fluorescent microscope (Fig. 3.11). Because of the low initial MOI (0.05), fibroblasts treated with DMSO medium showed a low level of infection, as expected (green staining; Fig. 3.11a, upper panel). However, cells treated with TSA medium showed an obvious increased number of cells expressing IE72/86 after infection (Fig. 3.11b, upper panel). Indeed, on average a five to six-fold increase in the number of cells showing IE72/86 expression occurred compared to the DMSO control. In order to preclude that this increase by TSA in permissiveness for IE gene expression was due to an increase in the level of virus entry, further analysis to determine the level of viral tegument protein, pp28, taken up during infection was carried out by Western blot analysis. No apparent difference could be seen in the amount of virus uptake between the DMSO (Fig. 3.11c, lane 1) or TSA (Fig. 3.11c, lane 2) treated cells. This result is also consistent with the findings of work on MCMV (Tang & Maul, 2003).

Indirect immunofluorescence analysis had already determined that TSA did lead to an increase in IE gene expression by increasing the number of cells initiating viral IE gene expression at a low MOI (0.05) independent of an increase in viral entry (Fig. 3.11). Consequently, further analyses at a range of MOIs (0.01-1) were carried out. Fibroblasts were again treated with either DMSO or TSA medium for 16 hours before infection with HCMV Toledo at a specified MOI. The cells were then fixed and stained for the presence of IE72/86 expression and the percentage of infected cells calculated from at least ten fields of view (Fig. 3.12). Consistently, the DMSO treatment of cells resulted in levels of IE72/86 expected for the MOI used. However, TSA treated cells consistently showed an increase in the percentage of cells staining positive for IE72/86 across the range of MOIs used. This result was most prominent at lower MOIs, where the significant difference has a P value of less than 0.01 and where the fold increase in IE72/86 positive cells is greatest. The fold change in IE72/86 positive cells resulting from TSA treatment gradually

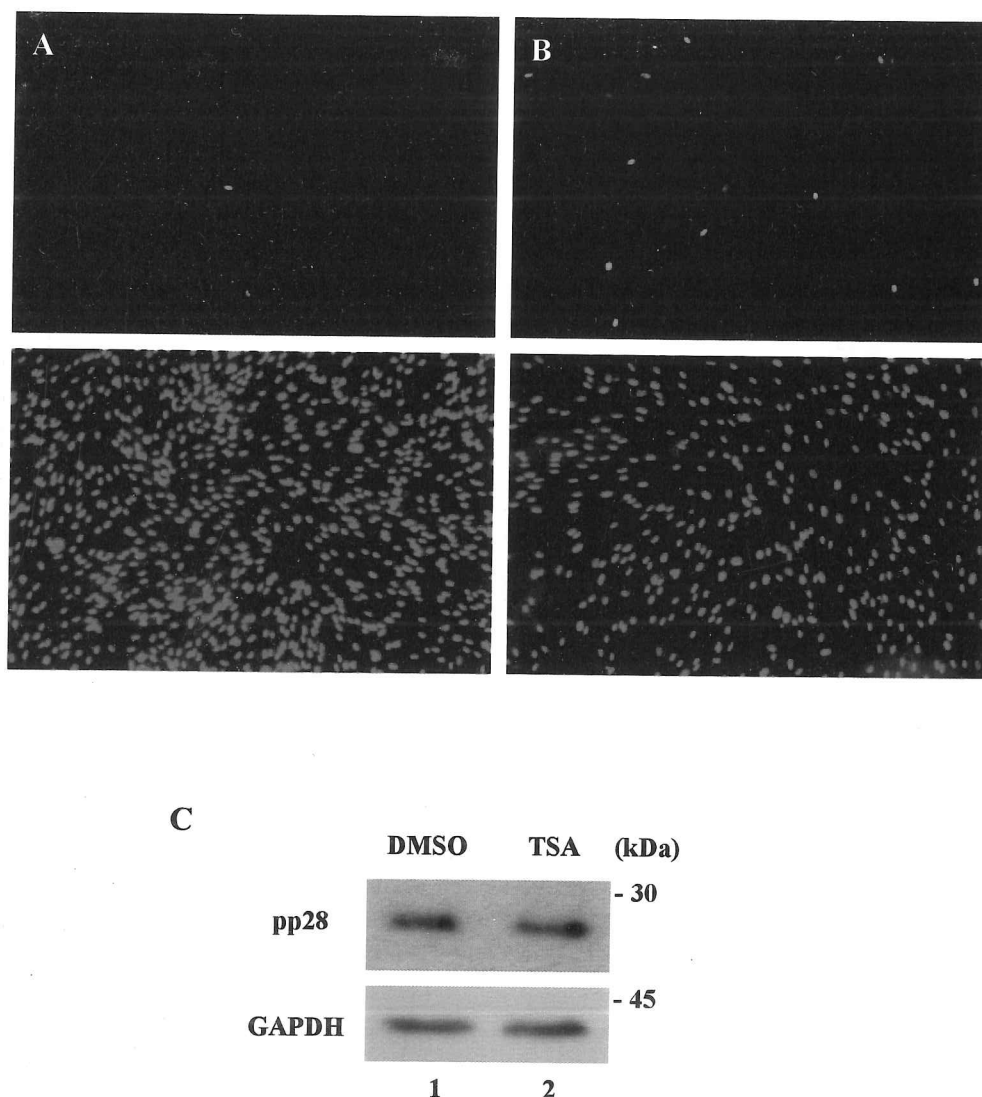


Figure 3.11 TSA treatment increases the permissiveness of lytic infection with HCMV at low multiplicities of infection independently of an increase in viral entry. Human fibroblasts were treated with either (A) DMSO or (B) TSA media. After 16 hours, cells were infected with HCMV Toledo (MOI 0.05) for 24 hours before cells were fixed and stained for IE72/86 (green) and Hoechst 33342 (blue) as a marker for cell nuclei. Analysis was carried out by fluorescent microscopy. (C) Human fibroblasts were treated with either DMSO (lane 1) or TSA (lane 2) media. After 16 hours, cells were infected with HCMV Toledo (MOI 0.5) for 6 hours before harvest and analysis of protein content by Western blot for the level of tegument protein (pp28) present, acting as a marker of viral entry. GAPDH staining was also carried out as a loading control.

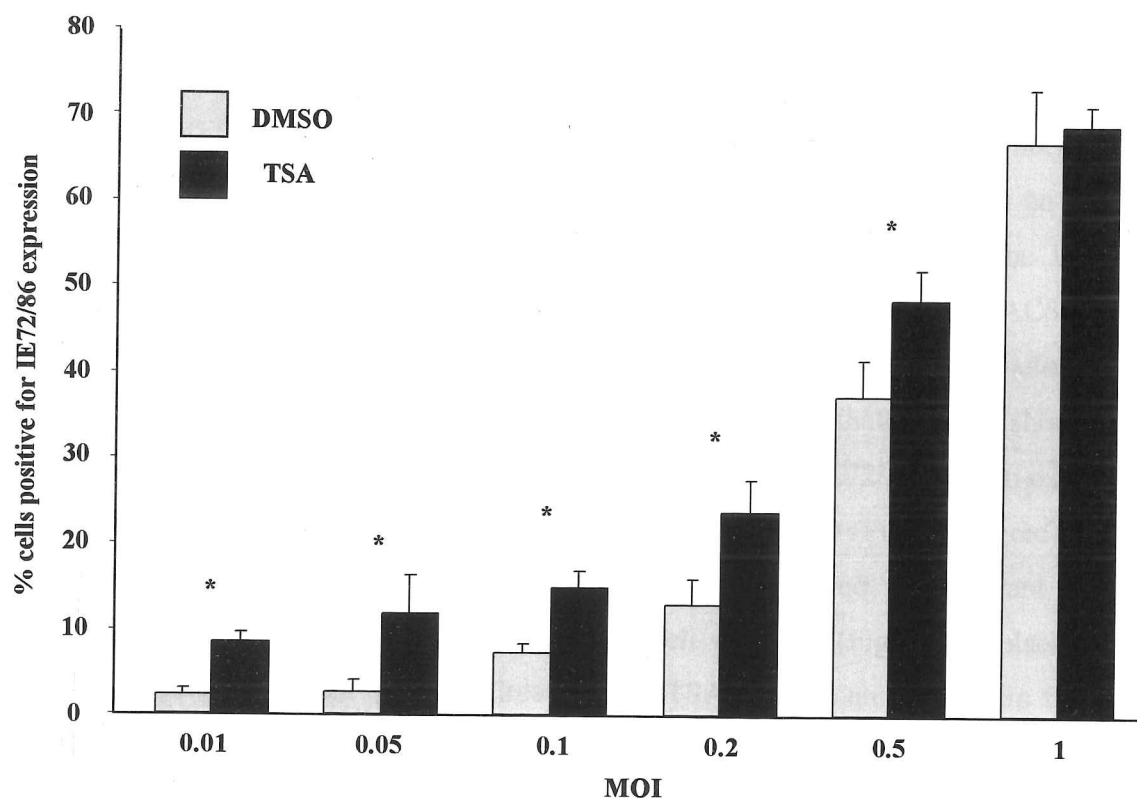


Figure 3.12 TSA treatment increases the permissiveness of lytic infection with HCMV at low multiplicities of infection. Human fibroblasts were treated with either DMSO (grey bars) or TSA (black bars) media. After 16 hours, cells were infected with HCMV Toledo at a range of MOIs (0.01 - 1) for 24 hours before cells were fixed and stained for IE72/86, using Hoechst 33342 as a marker for cell nuclei. Cells were then analysed by fluorescent microscopy with at least 10 fields of view counted per MOI, before the percentage of infected cells was calculated. Error bars, 1 S.D. of three independent experiments; * P value < 0.01.

decreases as higher MOIs are used, where little to no increase in IE72/86 expression by TSA is observed at an MOI of 1.

Although treatment of already permissive fibroblasts with TSA resulted in an increase in permissiveness of infection with HCMV at low MOIs (Fig. 3.12), it was still necessary to confirm whether TSA also increased the level of IE gene expression within an infected cell. Therefore, a further quantitative technique was utilised to analyse the levels of expression within individual cells: fluorescence activated cell sorting (FACS). Here, fibroblasts were treated with either DMSO or TSA medium for 16 hours before mock infection or infection with HCMV Toledo at a MOI of 3, such that all cells should have been infected. After 24 hours, cells were fixed and stained for IE72/86 protein expression before FACS analysis (Fig. 3.13). Fibroblasts treated with DMSO (Fig. 3.13a, red) or TSA (Fig. 3.13b, red) and then infected with HCMV Toledo showed predominant IE72/86 expression, compared to the uninfected treated cell controls (Fig. 3.13, black lines). However, infected cells that had been treated with TSA showed an increase in the mean fluorescence of the population (Fig. 3.13b, red) compared to those treated with DMSO (Fig. 3.13a, red). Consequently, TSA appears not only to increase the number of cells initiating IE gene expression when infected at low MOIs but also the level of viral IE gene expression in the infected cells as well.

3.2.5 TSA treatment causes increases in viral DNA replication, protein expression and infectious virus output

TSA pre-treatment of HCMV infected cells had clearly shown increases in the levels of IE gene expression, consistent with observations using MCMV infection of MEFs (Tang & Maul, 2003). Consequently, the next aim was to determine whether the use of TSA had further consequences on viral lytic infection. Since herpesvirus gene expression is controlled in a temporally regulated cascade (Fig. 1.5), ultimately dependent upon the expression of IE genes, an increase in IE gene expression may promote the levels of expression of subsequent viral genes. Therefore, the effects of TSA on the levels of early and late viral gene products, in addition to IE gene products, were assessed throughout the course of lytic infection using Western blotting (Fig 3.14). Fibroblasts were either DMSO or TSA treated for 16 hours and then infected with HCMV Toledo at a low MOI (0.1). Protein samples were then harvested at 6, 24, 48, 72 and 96 hours post-infection and levels

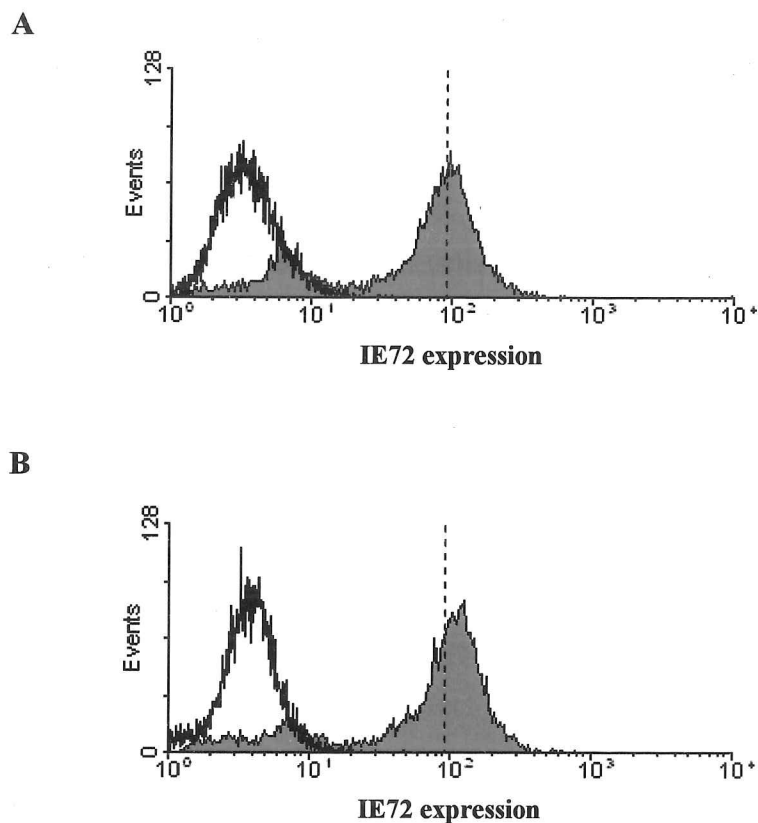


Figure 3.13 TSA treatment increases the level of viral gene expression within HCMV infected cells. Human fibroblasts were treated with either (A) DMSO or (B) TSA media. After 16 hours, cells were either mock infected (black line) or infected with HCMV Toledo (MOI 3; red) for 24 hours before cells were fixed, stained for IE72, washed and analysed by fluorescence associated cell sorting (FACS) analysis.

of viral protein expression were analysed. This included the major IE gene product IE72; the early UL44 gene product encoding the major DNA binding protein, p52, necessary for processivity of viral DNA replication (Anders & McCue, 1996); and the late gene product pp28 (UL99) – a tegument phosphoprotein essential for encapsidation of the genome and production of infectious virions (Spaete *et al.*, 1994; Silva *et al.*, 2003). Low levels of IE72 were observed in DMSO treated infected cells at 24 hours post-infection (Fig. 3.14, lane 2), as expected. However, as seen in Fig. 3.10, at this timepoint TSA treatment caused an increase in the level of IE gene expression (Fig. 3.14, lane 7). This increase in IE gene expression by TSA was observed throughout lytic infection right up to 96 hours post-infection (Fig. 3.14, lanes 8-10). Analysis of the early gene product, UL44, showed a similar increase in expression with TSA treatment of cells. Expression of UL44 in DMSO treated cells was detectable as early as 72 hours post-infection (Fig. 3.14, lane 4). However, a comparable level of expression was seen in TSA treated cells by 24 hours post-infection (Fig. 3.14, lane 7). Indeed, this effect of increased viral gene expression was also observed with the late gene product, pp28; TSA treated cells showed pp28 expression as early as 48 hours post-infection (Fig. 3.14, lane 8). Clearly, TSA treatment of fibroblasts prior to infection with HCMV caused an increase in the levels of gene expression of IE, early and late genes throughout the lytic cycle in already permissive cells.

The replication of viral DNA, for packaging into new virions, is entirely dependent on the expression of the prerequisite early viral gene products such as the UL44 coding protein, p52 (Anders & McCue, 1996). Since TSA clearly caused an increase in the levels of early gene expression upon infection with HCMV, an analysis of the effect of TSA on viral DNA replication was also undertaken (Fig. 3.15). Cells were again treated with either DMSO or TSA medium for 16 hours prior to infection with HCMV Toledo (MOI 0.5). DNA was extracted from cells at 6, 24, 48, 72 and 96 hours post-infection and specific PCR for the viral MIEP was then carried out. PCR products were then run out on 2% agarose gels and the relative levels of amplified products were quantified using Image J software, using GAPDH as a loading control. Levels of viral DNA are shown relative to input viral DNA quantified at 6 hours post-infection. The control DMSO treated cells (Fig. 3.15, grey bars) showed an increase in viral DNA levels at 48 hours post-infection, consistent with initiation of viral DNA replication. This increase then continued at both 72 and 96 hours post-infection, finally reaching a 4-fold increase in the level of viral DNA

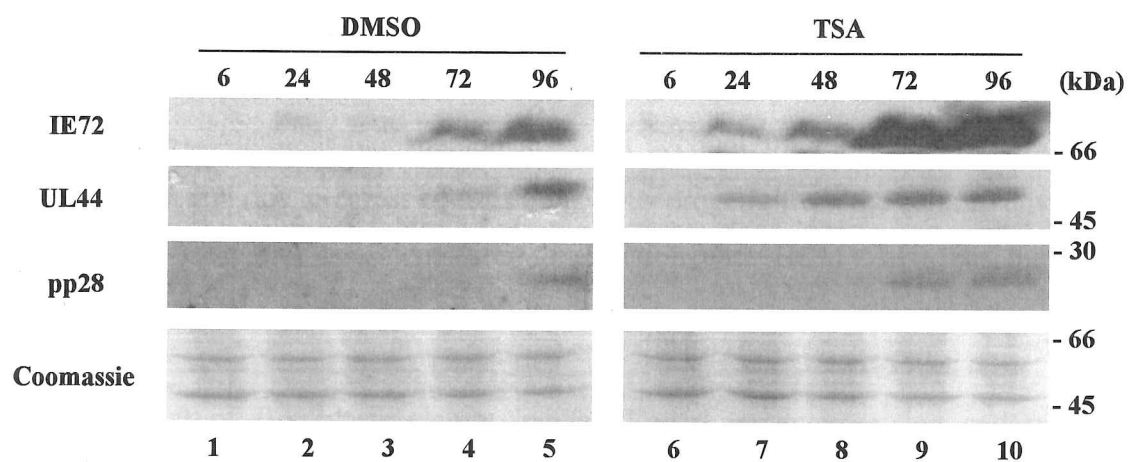


Figure 3.14 TSA treatment increases viral IE, early and late protein expression. Human fibroblasts were treated with either DMSO or TSA media. After 16 hours, cells were infected with HCMV Toledo (MOI 0.1) in fresh plain media and protein samples were taken at timepoints from 6–96 hours (lanes 1-5 & 6-10). Samples for each timepoint were run on 10% polyacrylamide gels before Western blotting for viral IE (IE72), early (UL44) and late (pp28) proteins. Coomassie blue staining was used as a loading control.

compared to the input level at 6 hours post-infection. In TSA treated cells, though, at 72 hours post-infection, the level of viral DNA detectable was increased by almost 3-fold and this trend continued through to the 96 hour post-infection timepoint. This result is also entirely consistent with the increase of early viral gene expression and production of early gene products, which viral DNA replication is ultimately dependent upon (Anders & McCue, 1996). Although this method does not account for the levels of viral genome escaping detection due to egress of infectious virus progeny from the cellular environment, it does show that TSA treatment prior to infection with HCMV also has the effect of increasing viral DNA replication.

TSA treatment of fibroblasts clearly increased viral gene expression and viral DNA replication. It was next asked whether this led to an increase in output of infectious virus, as has been seen with TSA treatment of MEFs prior to MCMV infection (Tang & Maul, 2003). Thus, the level of virus production from control or TSA treated cells was analysed. Fibroblasts were, again, treated with either DMSO or TSA and then infected with HCMV Toledo at a low MOI (0.01). Unadsorbed virus was then removed by washing twice with fresh EMEM-10 and the medium finally being replaced. Samples of the cell supernatant were then analysed every day for seven days and the output of virus from both populations of cells assessed by TCID₅₀ as well as the virus input used to infect both sets of cells (Fig. 3.16). From day 0 to day 3 post-infection, both DMSO (Fig. 3.16, grey diamonds) and TSA-treated cells (Fig. 3.16, black squares) showed little virus production, as expected, due to the eclipse period of the virus life cycle (Mocarski *et al.*, 2007). At 4 days post-infection, infectious virus was detectable in both control and TSA treated cells. However, by this timepoint TSA treated cells resulted in a 2-fold increase in the level of infectious virus progeny. Increased virus production from both populations of cells continued through the 5 and 6 day timepoints but TSA treated cells, again, showed further increases in infectious virus output, 3- and 4-fold respectively. However, the most prominent difference in virus output came at the 7 day post-infection timepoint when TSA treatment had caused a 14-fold increase in the total level of infectious virus output. Hence, TSA treatment of cells causes not only an increase in the levels of viral gene expression and subsequent DNA replication but also an increase in the total amount of infectious virus produced.

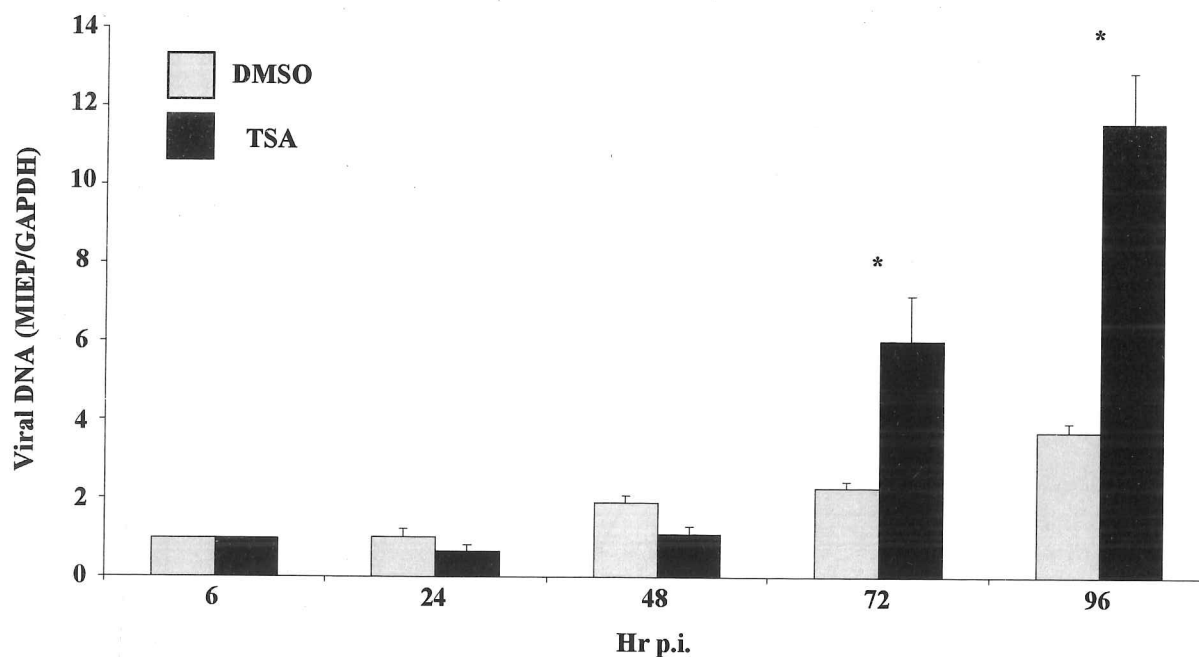


Figure 3.15 TSA treatment promotes viral DNA replication. Human fibroblasts were treated with either DMSO (grey) or TSA (black) media. After 16 hours, cells were infected with HCMV Toledo (MOI 0.5) in fresh plain media and DNA was extracted at timepoints from 6–96 hours. Both MIEP and GAPDH PCR was then performed on each sample, PCR products run on 2% agarose gels and the amount of viral DNA quantified by Image J analysis, expressed as a proportion of the input DNA (MIEP/GAPDH). Error bars, 1 S.D. of three independent experiments; * P value < 0.01.

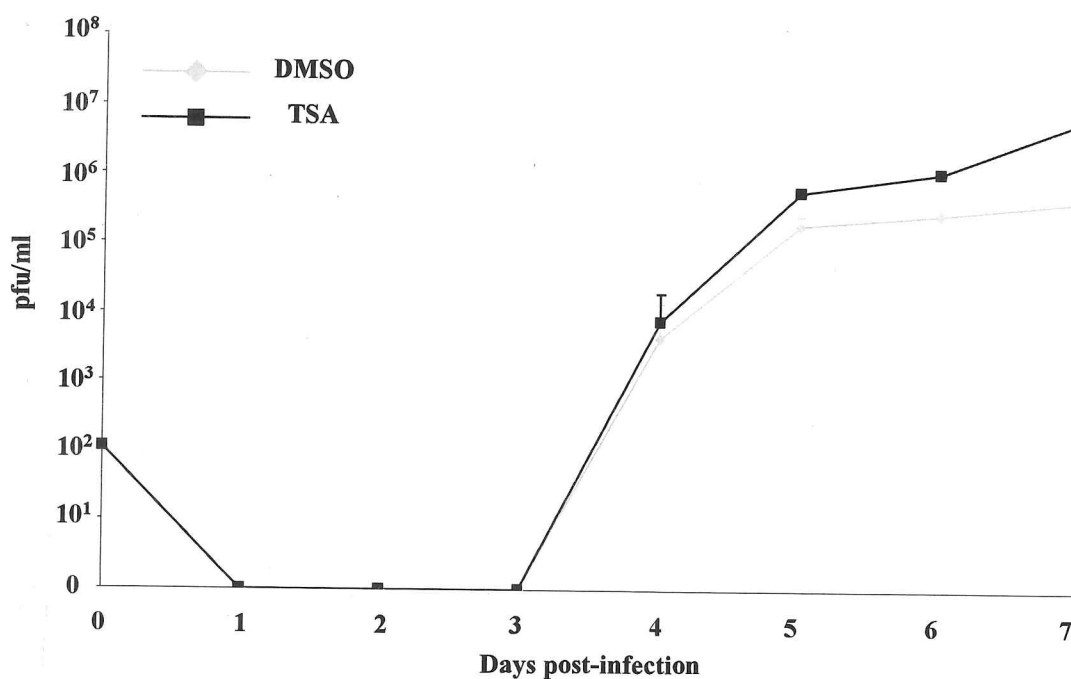


Figure 3.16 TSA treatment promotes the production of infectious virus. Human fibroblasts were treated with either DMSO (grey) or TSA (black) media. After 16 hours, cells were infected with HCMV Toledo (MOI 0.01) and supernatant samples were taken at timepoints for 7 days. Each sample was then used in serial dilutions to infect human fibroblasts and TCID₅₀ analysis carried out. The data is representative of three independent experiments. Error bars, 1 S.D.

3.2.6 TSA treatment causes changes to the chromatinisation of the MIEP at IE and early times of infection

Since TSA had led to substantial effects on HCMV gene expression and viral DNA replication, it was hypothesised that histone deacetylation must be involved in regulating viral gene expression. Consequently, ChIP analyses were carried out on fibroblasts either treated with DMSO or TSA for 16 hours to assess the viral MIEP with respect to the chromatin structure. Representative autoradiographs from the 3 hour and 24 hour post-infection samples are displayed (Fig. 3.17a & 3.19a). The intensity of amplified products from triplicate PCRs of each sample were then quantified using Image J and the data represented in chart format for both timepoints (Fig. 3.17b & 3.19b). As before, to ensure consistent immunoprecipitation of specific histone or histone associated proteins across each timepoint and throughout the full timecourse, a c-Fos PCR was carried out for both timepoints (Fig. 3.18 & 3.20).

At 3 hours post-infection with HCMV, the viral MIEP in DMSO treated cells was predominantly associated with H3K9me2 and HP1 β (Fig 3.17b, lane 2 & 3), with only low levels of acetylated histone-H4 (Fig. 3.17b, lane 1). This is consistent with repression of the MIEP immediately upon infection. In contrast, the MIEPs from TSA treated cells exhibited a very low level of association of H3K9me2 and HP1 β (Fig 3.17b, lane 5 & 6) but a much higher level of association with acetylated histone-H4 (Fig. 3.17b, lane 4). By 24 hours post-infection, the association of the MIEP with H3K9me2 and HP1 β in DMSO treated cells had diminished (Fig. 3.19b, lanes 2 & 3) with an increased recruitment of acetylated histone-H4 (Fig. 3.19b, lane 1). However, in stark contrast to this, TSA treated cells demonstrated much lower levels of association with H3K9me2 (Fig. 3.19b, lane 5) and acetylated histone-H4 with the MIEP (Fig. 3.19b, lane 4) and showed an increased association of HP1 β (Fig. 3.19b, lane 6). The control PCR for c-Fos used throughout the timecourse provided evidence that immunoprecipitation had occurred consistently throughout the analysis of 3 and 24 hour post-infection samples (Fig. 3.18b & 3.20b, respectively).

3.3 Discussion

It is now increasingly clear that latency and reactivation of HCMV is maintained and regulated, at least in part, by chromatin structure of the viral MIEP. For instance,

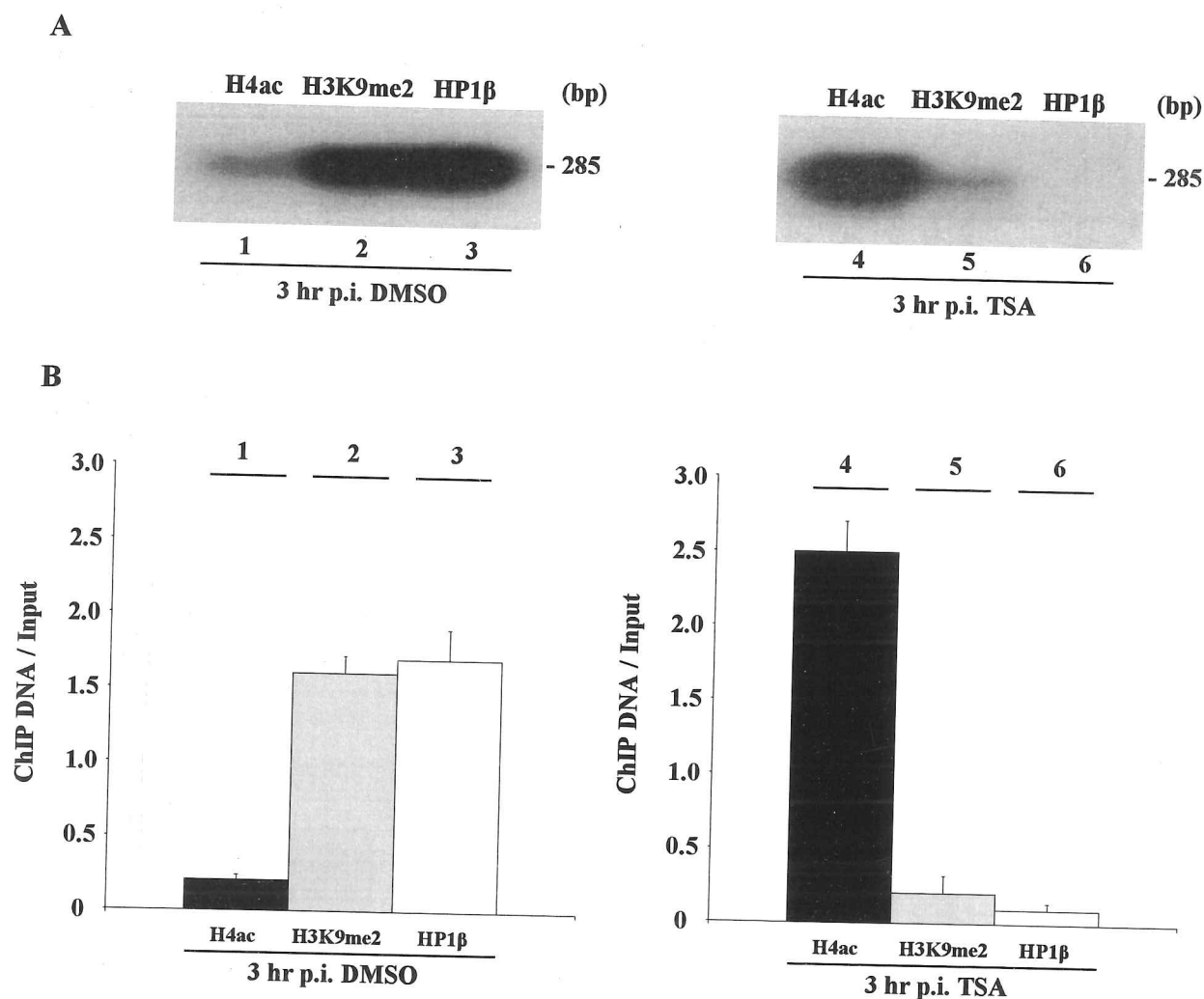


Figure 3.17 TSA treatment causes changes to the chromatinisation of the MIEP at 3 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, treated with either DMSO or TSA medium at 3 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1β antibody (HP1β, lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a MIEP-specific PCR and products run on a 2% agarose gel before Southern blot analysis. (B) Image J analysis was then used to quantify autoradiograph bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

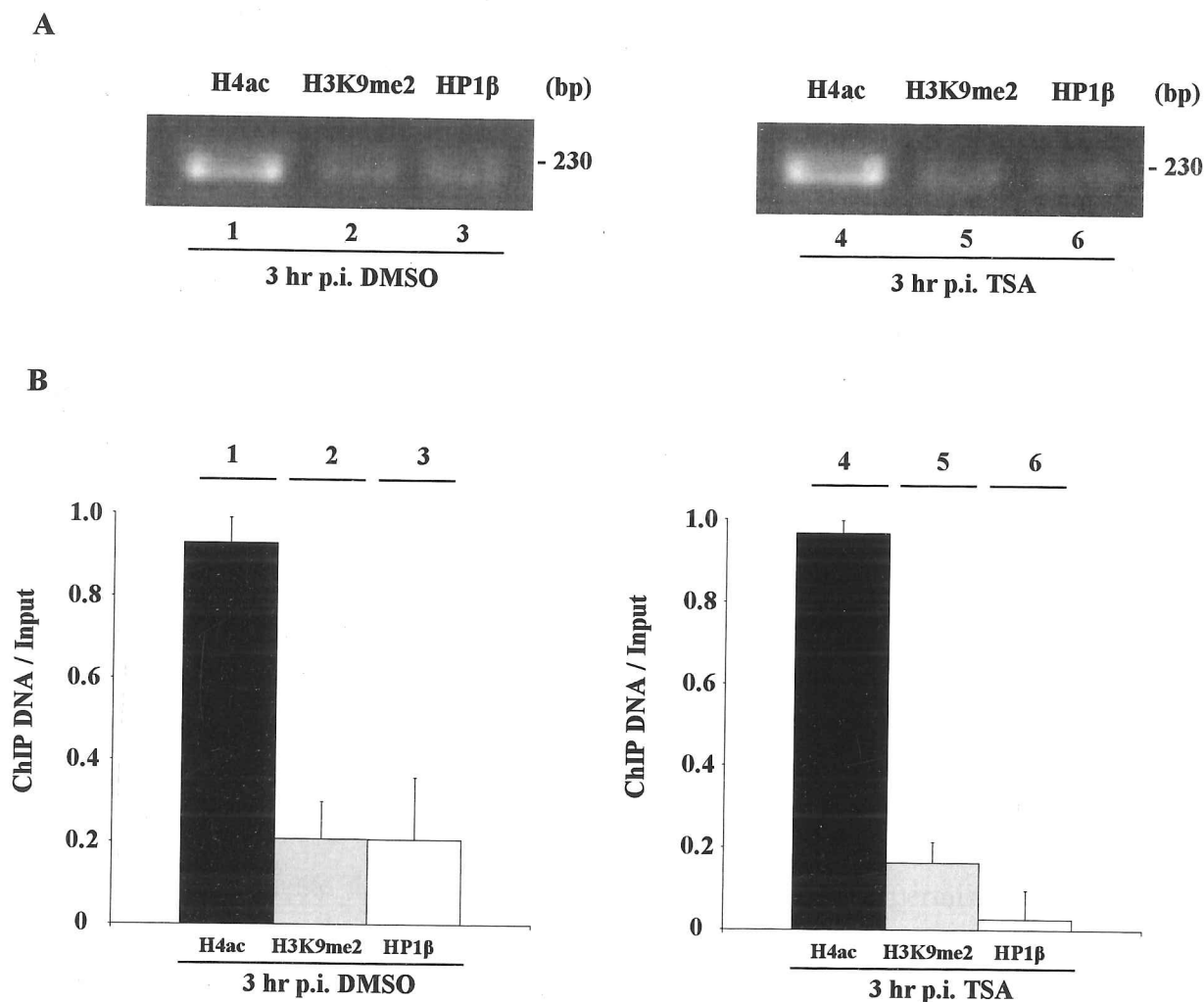


Figure 3.18 TSA treatment does not affect c-Fos chromatinisation at 3 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, treated with either DMSO or TSA medium at 3 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1 β antibody (HP1 β , lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a c-Fos-specific PCR and products run on a 2% agarose gel before visualisation using UV light. (B) Image J analysis was then used to quantify bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

chromatin remodelling of the MIEP of HCMV clearly regulates viral gene expression following experimental infection of primary myeloid cells and model cell lines conditionally permissive for IE gene expression. Upon infection of T2 cells, THP1 cells, undifferentiated monocytes and CD34⁺ myeloid progenitors there is a repressive chromatin structure around the MIEP consistent with a lack of viral IE gene expression in these cells. In contrast, infection of T2RA cells, macrophages and mature DCs results in the viral MIEP becoming associated with markers of transcriptionally active chromatin, consistent with their permissiveness for IE gene expression (Murphy *et al.*, 2002; Reeves *et al.*, 2005a; Ioudinkova *et al.*, 2006). Indeed, endogenous genomes present within CD34⁺ cells from a healthy seropositive individual when differentiated to mature DCs have also been shown to remodel the MIEP in the same way (Reeves *et al.*, 2005b). Thus, there is an intrinsic link between the differentiation state of cells, their permissiveness for IE gene expression and the specific pattern of chromatinisation of the MIEP upon infection. However, how changes in the chromatin structure of the viral MIEP might impinge upon infection in fully permissive cells is not known.

In order to investigate this, human fibroblasts, which are fully permissive for HCMV infection (McDonough & Spector, 1983) were used to examine the chromatin state of the viral MIEP during productive infection. It was clear that the MIEP was chromatinised throughout infection but the specific association of markers of transcriptionally active or repressed chromatin appeared consistent with the known temporal regulation of major IE gene expression at early (24 hours) and late (96 hours) timepoints. At 24 hours post-infection the MIEP was associated predominantly with acetylated histone-H4, consistent with transcription at this time. By 96 hours post-infection, recruitment of HP1 β was observed, consistent with the auto-regulation of the MIEP at late times of infection by the major IE gene product IE86 (Reeves *et al.*, 2006). However, a significant level of acetylated histone-H4 was still demonstrated: this may have been due to the chromatinisation of newly synthesised viral genomes at this time, although without the use of a viral DNA synthesis inhibitor such as PFA, this remained untested at this time (see Chapter 6). A low level of H3K9me2 present at viral MIEPs at this time was also apparent. However, this may have been due to high levels of HP1 β that would mask the presence of di- and tri-methylated lysine 9 of histone-H3 due to specific deposition at this site (Bannister *et al.*, 2005). Pleasingly, though, these data fit with that of Reeves *et al.* (2006)

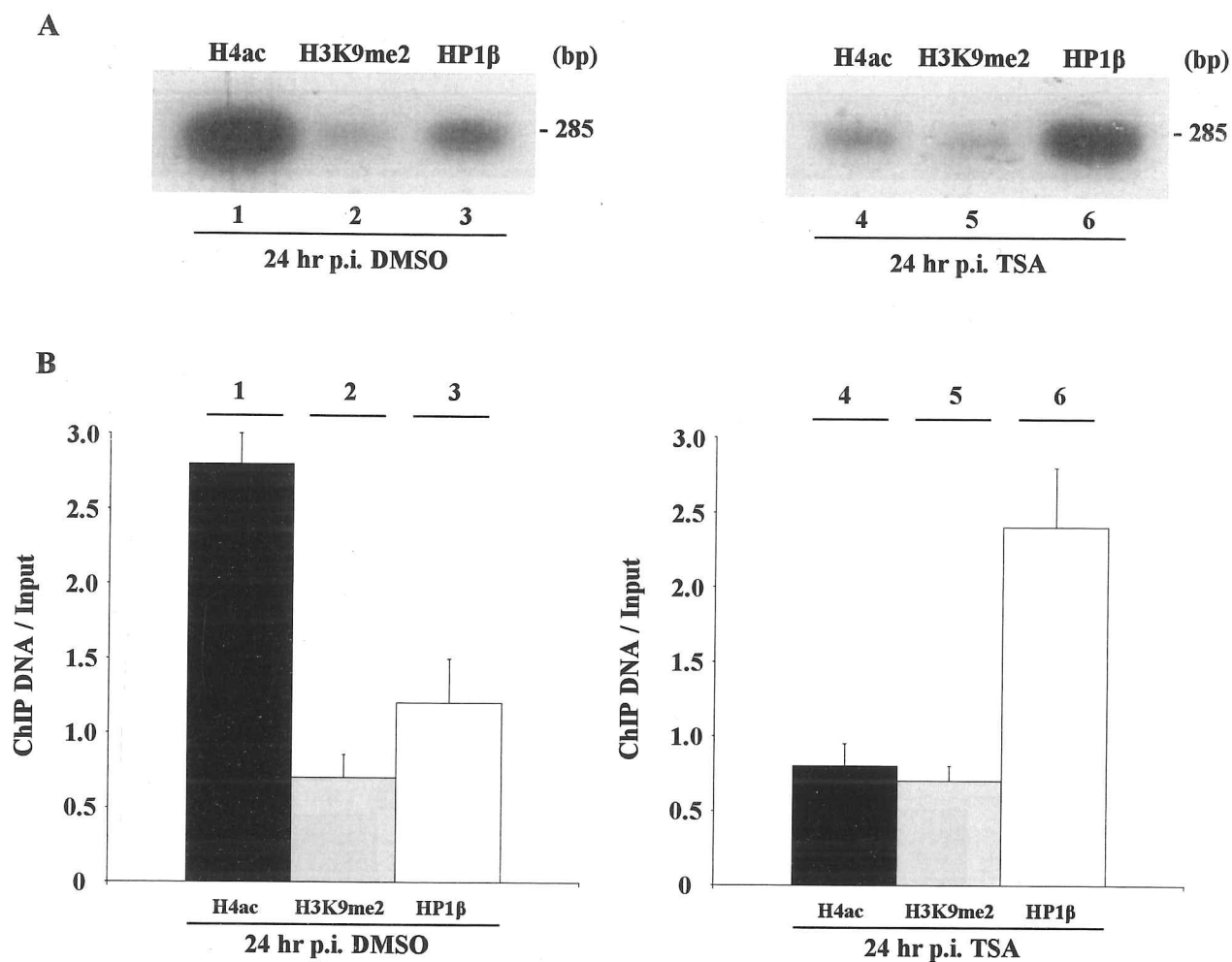


Figure 3.19 TSA treatment causes changes to the chromatinisation of the MIEP at 24 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, treated with either DMSO or TSA medium at 24 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1β antibody (HP1β, lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a MIEP-specific PCR and products run on a 2% agarose gel before Southern blot analysis. (B) Image J analysis was then used to quantify autoradiograph bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

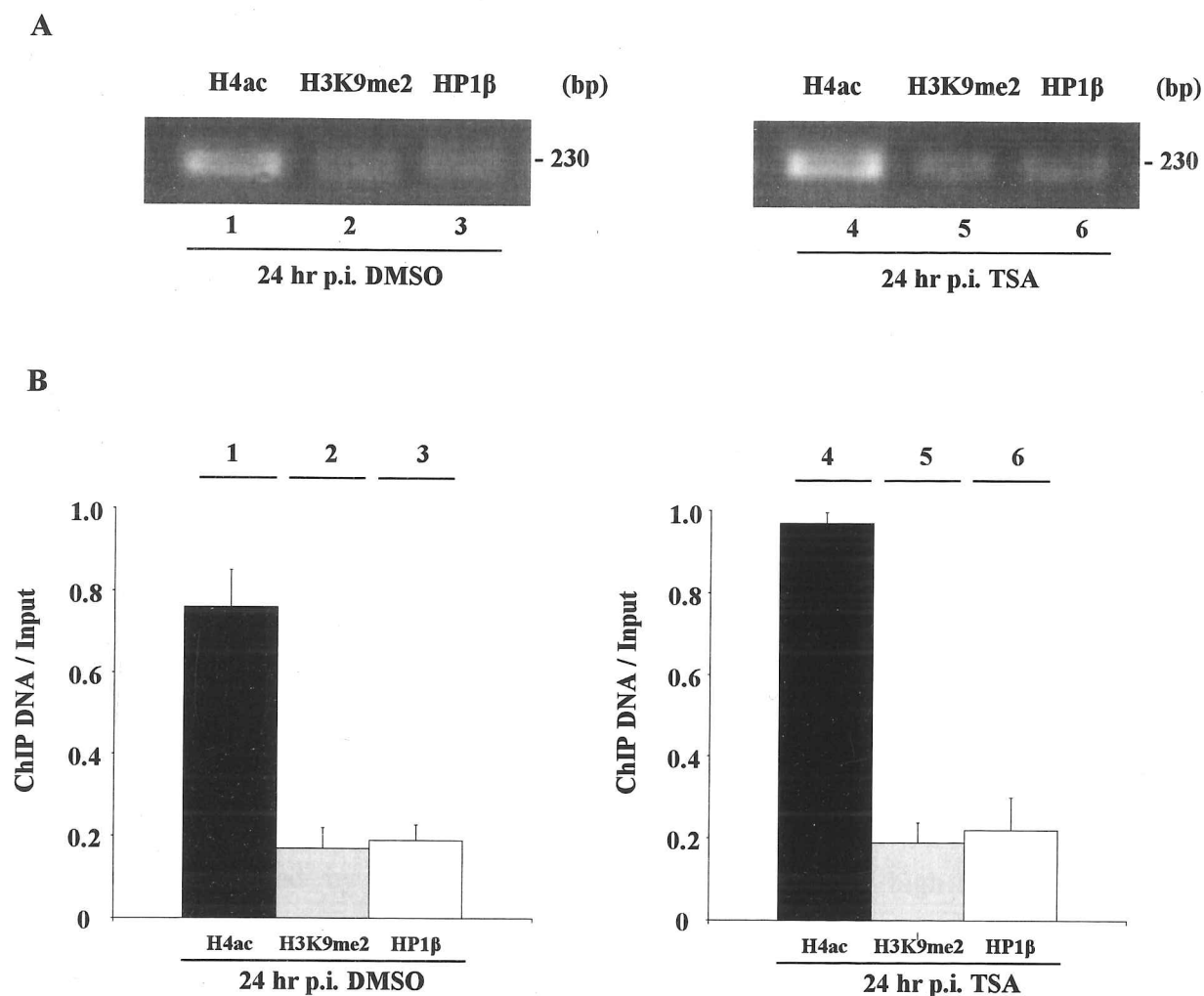


Figure 3.20 TSA treatment does not affect c-Fos chromatinisation at 24 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, treated with either DMSO or TSA medium at 24 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1 β antibody (HP1 β , lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a c-Fos-specific PCR and products run on a 2% agarose gel before visualisation using UV light. (B) Image J analysis was then used to quantify bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

where fibroblasts had been infected with HCMV Towne and ChIP assays conducted across a 24 to 96 hour timecourse. More recently, a further report using acetylated histone-H4 as a marker of promoter activation has also provided evidence of the temporal association of this marker with the MIEP, consistent with data presented here (Park *et al.*, 2007b).

Intriguingly, however, the chromatin status of the MIEP at 3 hours post-infection did not appear as expected for an IE timepoint. The viral MIEP was associated with high levels of H3K9me2 and HP1 β consistent with repression of the promoter at this time. Therefore, despite the use of fully permissive fibroblasts for this study, the viral genome appeared to be chromatinised in a repressed form at this timepoint. To rule out that this immediate association of repressive chromatin with the MIEP could be due to packaging of synthesised genomes in this form, gradient purified virions were prepared for protein analysis via Western blot. Analysis of the levels of histone-H3 specifically in virion preparations, compared to crude virus preparations or cellular material, found no histone-H3 associated with purified virions. In addition, these virions remained infectious after the purification process. The absence of histones associated with purified HCMV virions has also been suggested by other studies, including the use of high specificity mass spectrometry (MS)-based approaches (Baldick & Shenk, 1996; Varnum *et al.*, 2004). Additionally, more recent data suggests that the HSV-1 virion also contains no histone proteins (Ho & Fraser, 2008). Therefore, it is likely that the packaged HCMV genome is not associated with histone and that the repressive chromatin found at the MIEP immediately upon infection forms after release of the genome into the nucleus. Indeed, the assembly process of histones and nucleosomal structure onto naked infecting viral DNA has been observed before in studies using SV40 virus (Stillman, 1986; Sugasawa *et al.*, 1990) and recruitment of histones to viral DNA may represent an automatic cellular response to nascent DNA and provide explanation for the immediate chromatinisation of the viral MIEP upon infection of cells.

Throughout the study, specific PCR of the c-Fos promoter had been used as a control for examination of consistent immunoprecipitation of chromatin. This promoter was employed as it had previously been used as a similar control during HCMV infection of permissive MRC5 (embryonal lung) cells (Nevels *et al.*, 2004b). In this study, during the first 12 hours of infection, there was no change in the levels of acetylated histone-H4 at the c-Fos

promoter. However, it had also previously been published that HCMV can substantially increase the level of c-Fos expression during infection of human fibroblasts by 12 hours and that the promoter in transfected cells could be trans-activated by IE gene expression (Hagemeier *et al.*, 1992b). In contrast, infection of human embryonal lung cells at an MOI of 10 had been found to increase c-Fos transcription almost 5-fold after 40 min but that expression returned to basal levels by 2 hours (Boldogh *et al.*, 1990a). Although the previous findings with fibroblasts argued against the use of the c-Fos promoter as a control for ChIP analysis of the viral MIEP, the further data fully supported its use from a 3 hour post-infection timepoint. Indeed, the results presented here also show very little change in the chromatin profile of the c-Fos promoter throughout the full timecourse of productive infection and thus it was validated as a robust control for the ChIP analyses presented.

The chromatinisation of the viral MIEP appeared to occur very rapidly upon infection, with histone proteins detectable by ChIP analysis as early as 3 hours post-infection. However, intriguingly, at this time the MIEP appeared to be predominantly associated with hallmarks of repressed chromatin structure: H3K9me2 and HP1 β . For repressive chromatin structure to be established, it is likely that chromatin remodelling enzymes such as HDACs would be involved. Therefore, to address whether this was the case, the HDAC inhibitor, TSA, was employed. Treatment of cells with TSA prior to infection with HCMV led to an increase in the production of IE RNA and protein in infected fibroblasts as well as in U373 cells due to an increase in the number of cells initiating IE gene expression but also increases in levels of IE gene expression within each infected cell. This was not due to TSA-mediated increases in virus uptake as determined by incoming levels of pp28 tegument protein with similar observations having been made during MCMV infection (Tang & Maul, 2003).

Treatment of fibroblasts with TSA prior to infection with HCMV also resulted in increases in gene expression of all classes of viral genes: IE, early and late. The consequence of increases in early and late gene expression could be due, in part, to the initial increase in major IE gene expression whereby transactivation of the early and late classes of genes would occur earlier due to earlier accumulation of IE protein. However, the increases in early and late gene expression could also be explained more directly if the promoters of these genes were also chromatinised. This hypothesis will be tested and discussed in

Chapter 6. Nevertheless, the ability of TSA to increase expression of all classes of viral genes could result from a shortening of the infection cycle due to earlier and more efficient IE gene expression. Subsequent semi-quantitative PCR analysis of genome number throughout infection provided evidence to suggest that this was indeed occurring. Viral DNA synthesis was increased upon TSA treatment of infected fibroblasts and was concomitant with a 10-fold rise in the number of infectious virus particles produced after seven days post-infection. This finding is, again, consistent with similar studies carried out with MCMV (Tang & Maul, 2003).

The effects of TSA treatment on HCMV IE gene expression and productive cycle suggested that chromatin structure and, in particular, repression of the viral MIEP may occur immediately upon infection. To correlate this to the state of chromatin of the MIEP, ChIP analysis of the MIEP in fibroblasts was carried out. At 3 hours post-infection in control cells, the MIEP was associated with acetylated histones but predominantly methylated histones and HP1 β . However, at the same timepoint in TSA treated fibroblasts, the MIEP was predominantly associated with acetylated histone-H4. This finding is consistent with the inhibition of HDAC activity by TSA resulting in increases in the levels of viral IE gene expression and subsequent early and late gene expression and virus production. The association of acetylated histones with the MIEP at 24 hours post-infection in infected control cells is entirely consistent with levels of viral IE gene expression at this time. However, intriguingly, in TSA treated cells at the same timepoint, the viral MIEP was heavily associated with HP1 β . Data from this timepoint may at first appear contradictory to the RNA and protein level data, as obvious increases in IE gene expression were demonstrated at this time. One explanation for this could be the relative stability of the IE72 protein. Thus, protein levels would reflect the accumulation of IE gene product over the full 24 hour period. Therefore, the increase in gene expression throughout infection with TSA treatment is directly related to an earlier association of the viral MIEP with acetylated histone-H4 and most likely due to earlier initiation of viral IE gene expression. Additionally, the high association of the MIEP with HP1 β at 24 hours post-infection with TSA treatment may be, in part, due to the earlier recruitment of HMTs by IE86 and auto-repression of the MIEP due to the shortening of the viral productive cycle resulting from earlier initiation of IE gene expression in TSA treated cells.

The use of c-Fos promoter specific PCR throughout this study controlled for any variability in immunoprecipitation of histones and histone associated proteins with the viral DNA. It is known that TSA does increase c-Fos transcription levels through increasing pre-initiation complex recruitment to the promoter (Fass *et al.*, 2003). However, this published effect is only minor and, as the promoter is already constitutively active, does not appear to affect the chromatin structure of the c-Fos promoter significantly.

Overall, the data presented in this chapter illustrate that the MIEP of HCMV is chromatinised throughout productive infection of permissive fibroblasts. Immediately upon infection, the MIEP is repressed due to chromatinisation which is at least in part mediated by HDACs. TSA treatment is able to abrogate this repression at the level of the chromatin status of the MIEP causing increases in viral gene expression and ultimately production of infectious virions.

4. Results

hDaxx mediates HCMV IE gene expression repression through regulation of the chromatin structure of the MIEP

4.1 Introduction

The results presented in Chapter 3 had established that the MIEP of HCMV was temporally associated with chromatin structure which was remodelled throughout productive infection of human fibroblasts. Intriguingly, however, despite these cells being fully permissive for lytic infection, repressive forms of chromatin were found to be associated with the promoter at very IE times of infection. The use of the HDAC inhibitor TSA relieved this initial repression leading to changes in viral gene expression, replication and progeny virus output as well as chromatin structure at the MIEP. Therefore, HDACs are likely to play a major role in the recruitment of repressive chromatin to the MIEP upon infection. Interestingly, it is known that herpesvirus genomes, upon entry to the nucleus of an infected cell, are deposited at ND10 (Maul, 1998). ND10 are known to recruit HDACs and HMTs, and thus could promote an environment of repression at which the viral genome locates to (see section 1.11). However, chromatin remodelling enzymes do not act indiscriminately and are not targeted to ND10 alone: they rely upon interaction with other proteins for specificity of location. As explained earlier (see section 1.11), ND10 are composed of several cellular proteins (Negorev & Maul, 2001). In addition to the PML protein, the defining component of ND10, many other ND10-associated proteins are known to have transcriptional control properties. One such protein is Daxx.

Daxx was initially identified as a novel Fas-binding protein by two-hybrid screening (Yang *et al.*, 1997) and is now known to interact with a range of cellular and viral proteins in a number of different key cell signalling pathways. Initially, the protein was suggested to be involved in the control of apoptosis, as transient over-expression studies using murine Daxx (mDaxx) demonstrated increases in the level of Fas-mediated apoptosis within certain cell types (Yang *et al.*, 1997; Torii *et al.*, 1999). However, conversely, transient over-expression of a truncated dominant-negative form of mDaxx was found to inhibit Fas-mediated apoptosis (Torii *et al.*, 1999). mDaxx has also been reported to act as a cytoplasmic Fas-receptor adaptor molecule able to recruit the apoptosis signal-related

kinase ASK1 (Chang *et al.*, 1998) that can phosphorylate and activate transcription factors such as c-Jun (Kyraiakis & Avrush, 1996; Yang *et al.*, 1997). Further, mDaxx has also been postulated to act as a dominant-negative inhibitor of TGF β -induced apoptosis in hepatocytes (Perlman *et al.*, 2001). In contrast, studies using a dominant interfering human Daxx (hDaxx) have shown that hDaxx plays no physiological role in FasL-mediated JNK signalling and apoptosis (Villunger *et al.*, 2000). Furthermore, it has been shown that cells subjected to siRNA technology to Daxx increase levels of apoptosis, therefore making it improbable that Daxx promotes Fas-induced apoptosis in this system (Michaelson & Leder, 2003).

hDaxx resides primarily within the nucleus likely due to two putative nuclear localisation sequences (Pluta *et al.*, 1998) and is a known component of ND10 (see section 1.11.3). Recruitment of hDaxx to ND10 is mediated through interaction with PML, requiring sumoylation of PML by SUMO-1 (Ishov *et al.*, 1999). However, when not localised at ND10, hDaxx is associated with condensed, transcriptionally inactive chromatin (Li *et al.*, 2000a; Hollenbach *et al.*, 2002). The transcriptional repressive properties of hDaxx are consistent with four structural domains commonly found in other repressors: two predicted paired amphiphatic helices, an acid rich domain and a Ser/Pro/Thr rich domain (Hollenbach *et al.*, 1999). hDaxx has been shown to have inhibitory effects on Pax3-mediated transcription and the ability to interact with ETS1 and repress transcription mediated by this proto-oncogene (Hollenbach *et al.*, 1999; Li *et al.*, 2000b; Lehembre *et al.*, 2001). Additionally, hDaxx has also been found to mediate suppression of the glucocorticoid, mineralcorticoid and androgen receptors, p53 family proteins, Smad4 and NF- κ B predominantly through differential use of its SUMO-interacting motif and also IFN-induced STAT3 signalling (Lin *et al.*, 2002; Kim *et al.*, 2003; Lin *et al.*, 2003; Lin *et al.*, 2004a; Obradovic *et al.*, 2004; Zhao *et al.*, 2004; Chang *et al.*, 2005; Croxton *et al.*, 2006; Muromoto *et al.*, 2006; Park *et al.*, 2007a; Shih *et al.*, 2007). However, in B cells, hDaxx was found to be able to either activate or repress Pax5-mediated transcription depending upon the cell line used and that activation via hDaxx was dependent upon the recruitment of the HAT CBP (Emelyanov *et al.*, 2002).

Although much is known of the multifunctional roles of hDaxx, the mechanism by which hDaxx is able to mediate repression has remained elusive. However, observations that

hDaxx can interact with HDACs and chromatin has shed some light on this. Dependent upon the post-translational phosphorylation state of the protein, Daxx has been demonstrated to interact with various chromatin remodelling enzymes including HDAC1, HDAC2, DNA methyltransferase 1 (DNMT1) and α -thalassaemia/mental retardation syndrome X-linked (ATRX); ATRX can also interact with HP1 as well as core histone proteins and the chromatin binding protein Dek (Hollenbach *et al.*, 2002; Li *et al.*, 2000a; Ishov *et al.*, 2004; Muromoto *et al.*, 2004; Waldmann *et al.*, 2004; Puto & Reed, 2008). It was further hypothesised that the association of the HDAC2/hDaxx complex and its ability to bind to chromatin, through such proteins as Dek, then leads to the deacetylation of histones upon interaction with chromatin and the subsequent repression of transcription (Hollenbach *et al.*, 2002). Furthermore, it has also more recently been reported that hDaxx is able to bind to SUMO-modified CBP and modulate CBP transcriptional control and that the expression of the c-met proto-oncogene is controlled by chromatin structure mediated by hDaxx and HDAC2 recruitment (Kuo *et al.*, 2005). Therefore, hDaxx appears to be a distinct transcriptional repressor of various cellular genes that is also able to locate to ND10. As such, hDaxx has acquired some attention from those investigating the control of herpesvirus gene expression.

The possibility that hDaxx may play some role in the immediate repression of HCMV IE gene expression upon infection by recruiting repressors to modulate the chromatin structure of the MIEP has recently gained support through studies on the transactivation properties of HCMV tegument phosphoprotein pp71. Encoded by UL82, pp71 is loaded into the virion before egress of the progeny virus and as such is delivered to the cell upon entry (see Fig. 1.4). As well as forming a structural component of the virus, it has long been known that pp71 acts as a transactivator of the MIEP during HCMV lytic infection (Liu & Stinski, 1992; Schierling *et al.*, 2004). Further, pp71 has been shown to transduce gene expression from the MIEP in recombinant HSV-1 mutants; although, pp71 does not cause this through disruption of ND10, as seen with IE72, which is unnecessary for this activity (Homer *et al.*, 1999; Marshall *et al.*, 2002; Preston & Nicholl, 2005). Interestingly, the mechanism by which pp71 is able to locate to ND10 is actually via interaction with a coiled-coil domain of the hDaxx protein which is then targeted to ND10 via recruitment by sumoylated PML (Hofmann *et al.*, 2002; Ishov *et al.*, 2002). This mechanism, although surprising due to the repressive qualities of hDaxx, has been confirmed through work

showing that pp71 mutants unable to bind to hDaxx were not able to induce IE gene expression (see Fig. 1.12) (Cantrell & Bresnahan, 2005). In addition, intriguingly, it has further been established that the presence of pp71 during HCMV infection is both necessary and sufficient for proteasomal degradation of hDaxx during HCMV infection (Saffert & Kalejta, 2006).

Since pp71 appears to target hDaxx for proteasomal degradation during HCMV infection, it has been hypothesised that hDaxx, when present at ND10, may play a role in the control of transcription of HCMV upon infection. Work performed in our laboratory has provided good evidence that hDaxx plays a fundamental role in the regulation of HCMV IE gene expression: stable over-expression of hDaxx in permissive U373 leads to a complete abrogation of herpesvirus lytic infection (Woodhall *et al.*, 2006). Since there is now good evidence that hDaxx is able to repress gene expression through association with chromatin remodelling enzymes, it was investigated whether the association of repressive chromatin with the MIEP immediately upon infection was mediated by hDaxx.

4.2 Results

4.2.1 TSA treatment causes de-repression of HCMV IE gene expression in infected hDaxx-stably expressing U373 cells

Since it had been established within the laboratory that normally permissive U373 cells stably expressing hDaxx as an RFP-hDaxx fusion protein (U373-RFP-hDaxx) were refractory to infection with HCMV, but that virus super-infection at high MOI could abrogate this repression (Woodhall *et al.*, 2006), it was first decided to investigate whether use of HDAC inhibitors could rescue virus gene expression in RFP-hDaxx expressing U373s. Initially, control cells stably transfected with the pDsRed-N1 construct (U373-RFP) and expressing RFP were treated with either DMSO or TSA media for 16 hours. Cells were then infected with HCMV Toledo (MOI 0.2) for 24 hours before cells were fixed, permeabilised and stained for IE72/86 expression with counter-staining of nuclei through Hoechst 33342. Staining was then viewed under a fluorescent microscope (Fig. 4.1a). U373-RFP cells treated with DMSO showed a low level of infection, as determined by expression of IE72/86 (green staining), which often co-localised with cells expressing RFP (Fig. 4.1a, left panel). U373-RFP cells treated with TSA showed an obvious increase in the number of cells expressing IE72/86 after infection whether expressing RFP or not

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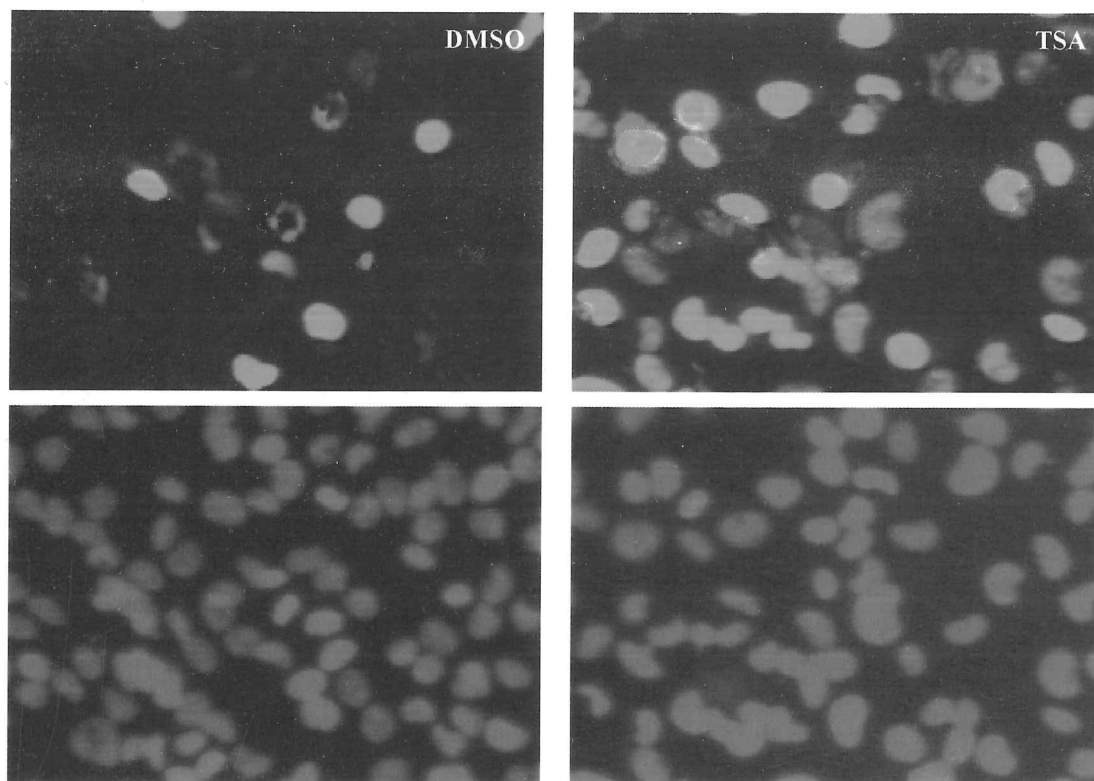
(Fig. 4.1a, right panel). To assess these mixed populations of cells quantitatively, the number of non-RFP or RFP-expressing cells co-expressing IE72/86 from at least 10 fields of view per experiment were counted (Fig. 4.1b). Both sub-populations of cells demonstrated approximately the same level of co-expression when treated with DMSO (Fig. 4.1b, grey bars). However, TSA treatment caused a 3- to 4-fold increase in the levels of IE gene expression in both RFP-expressing and non-expressing cells (Fig. 4.1b, black bars). Hence, U373-RFP cells routinely expressed IE genes upon infection but TSA was able to induce an increase in this level, consistent with TSA treatment of infected fibroblasts in Chapter 3.

The same analysis was then carried out on U373 cells stably expressing hDaxx (U373-RFP-hDaxx) (Fig. 4.2). Although DMSO treated cells not expressing RFP-hDaxx showed a similar level of infection to that seen with non-RFP cells within the U373-RFP population, RFP-hDaxx expressing cells, known to be refractory to IE gene expression, showed little to no expression of IE72/86 (Fig. 4.2a, left panel). With TSA treatment, non-RFP cells showed a clear increase in the number of IE72/86 expressing cells (Fig. 4.2a, right panel). However, TSA treatment of RFP-hDaxx expressing cells caused a clear increase in the level of IE72/86 and RFP-hDaxx co-staining (Fig. 4.2a, right panel). This data was again quantified as above (Fig. 4.2b). This demonstrates that DMSO and TSA treated non-RFP cells in this population behave similarly to those from the U373-RFP population, with an approximate 4-fold increase in IE72/86 staining (Fig. 4.2, grey bars). However, RFP-hDaxx expressing cells treated with DMSO show a much lower level of IE gene expression (0.3%) which can be increased over 100-fold by the use of TSA treatment (32%) (Fig. 4.2b, black bars). Hence, TSA treatment can result in the de-repression of IE gene expression caused by hDaxx over-expression.

4.2.2 The MIEP in infected hDaxx-stably expressing U373 cells is associated with repressive chromatin

Since TSA treatment of RFP-hDaxx expressing cells had resulted in de-repression of IE gene expression upon infection, it was hypothesised that, in the presence of stable hDaxx expression, the MIEP might be subject to more potent repression. To analyse this, ChIP analysis was carried out on control U373-RFP cells and U373-RFP-hDaxx cells. However, as the U373-RFP and U373-RFP-hDaxx cells were mixed populations with variable levels

A



B

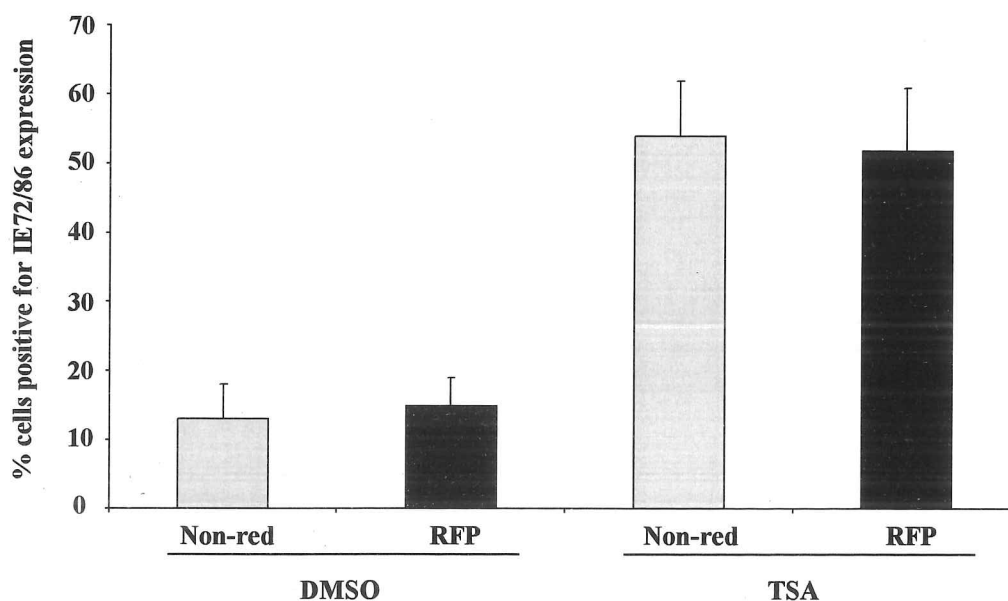
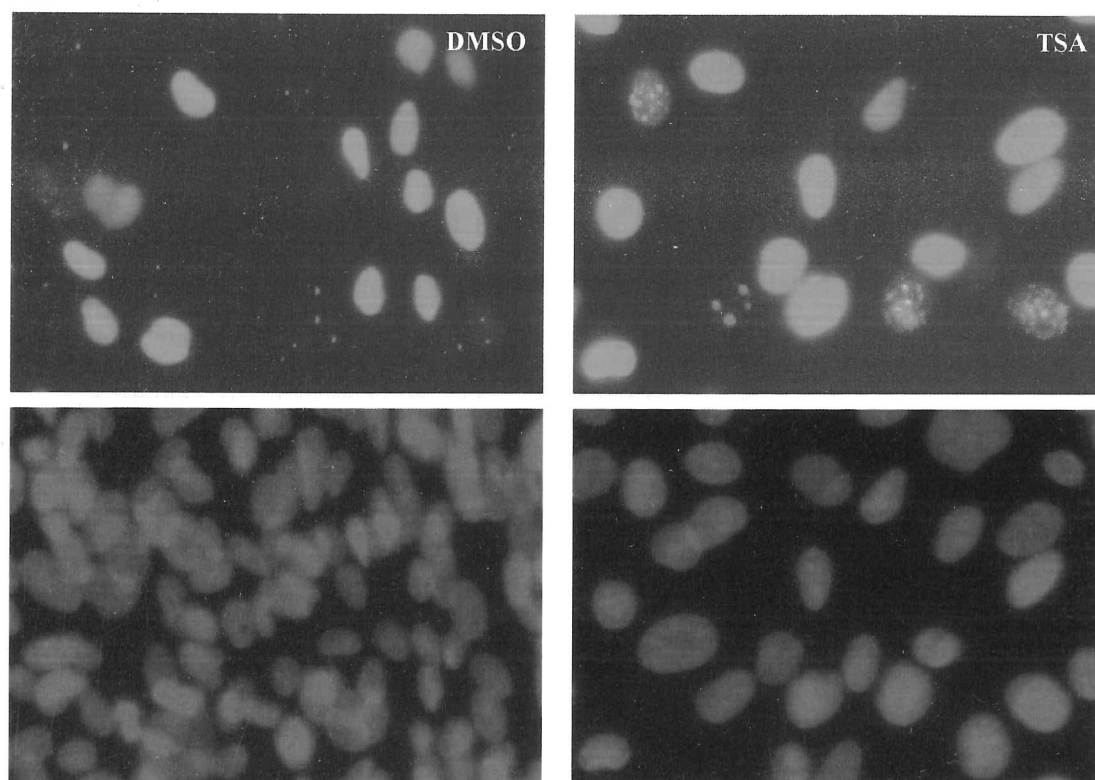


Figure 4.1 TSA causes an increase in permissiveness for infection of U373-RFP cells. U373-RFP cells, stably expressing RFP, were treated with either DMSO or TSA media for 16 hours. (A) Cells were then infected with HCMV Toledo (MOI 0.2) for 24 hours before cells were fixed and stained for IE72/86 expression. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei. (B) The number of RFP-expressing and non-red cells expressing IE72/86 was then quantified by fluorescent microscopy using at least 10 representative fields of view per experiment. Error bars, 1 S.D. of three independent experiments.

A



B

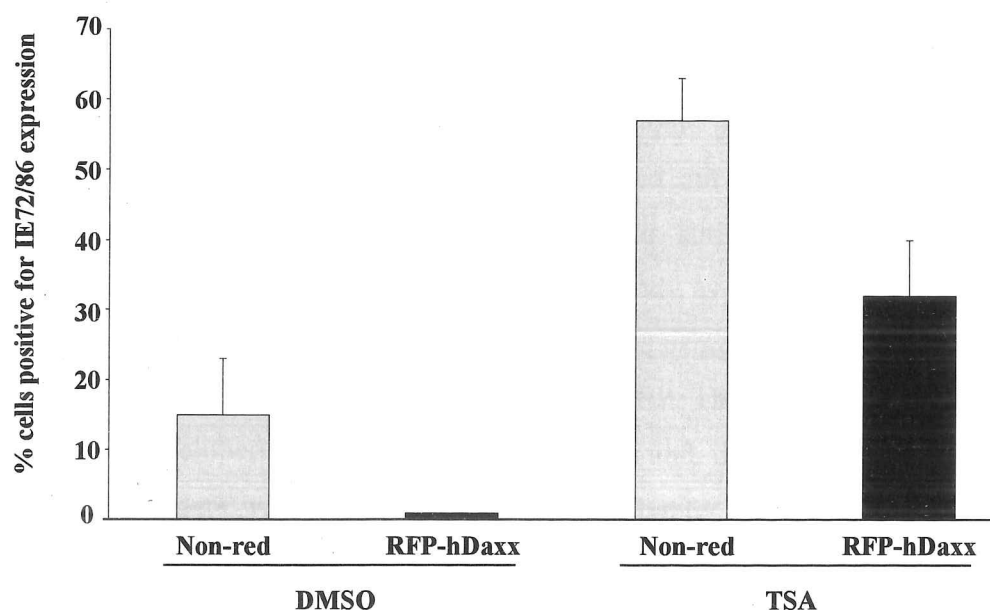


Figure 4.2 TSA causes de-repression of the HCMV IE gene expression in U373-RFP-hDaxx cells. U373-RFP-hDaxx cells, stably expressing hDaxx as an RFP fusion protein, were treated with either DMSO or TSA media for 16 hours. (A) Cells were then infected with HCMV Toledo (MOI 0.2) for 24 hours before cells were fixed and stained for IE72/86 expression. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei. (B) The number of RFP-hDaxx expressing and non-red cells expressing IE72/86 was then quantified by fluorescent microscopy using at least 10 representative fields of view per experiment. Error bars, 1 S.D. of three independent experiments.

of transgene expression (Fig. 4.3), it was decided to purify the RFP or RFP-hDaxx expressing cells from the mixed populations. Live sorting of both populations of cells was carried out using FACS to isolate the RFP or RFP-hDaxx expressing cells (Fig. 4.4). Analysis of the total populations of cells demonstrated that 27.78% of the U373-RFP population expressed RFP strongly and that 1.48% of the U373-RFP-hDaxx expressed RFP-hDaxx equivalently (Fig. 4.4b). These cells were then isolated from the population (Fig 4.4a, black boxes) and cultured with selection (G418 at 1mg/ml). After this time, the RFP and RFP-hDaxx expressing cells were subjected to further analysis to determine an estimate of RFP or RFP-hDaxx expression by fluorescent microscopy (Fig. 4.5). At least 10 fields of view were counted and this demonstrated that the level of RFP-positive cells within the U373-RFP population had been enhanced slightly to 43%. However, although the RFP-hDaxx positive sub-population of the U373-RFP-hDaxx cells was only increased to 36%, this was a considerable enhancement from the initial 1.48% and provided a similar proportion of RFP-positive cells to the control U373-RFP population. As such, ChIP analysis was then carried out on these populations.

U373-RFP and U373-RFP-hDaxx cells were first incubated for 48 hours with serum-free medium to synchronise the cells. Then, after this time, the cells were infected with HCMV in serum-containing medium at an MOI of 0.1. At 24 hours post-infection, the cells were fixed with formaldehyde (1%) and ChIP assays carried out using either control serum (Con), an anti-acetylated histone-H4 antibody (H4ac), an anti-di-methyl lysine 9 histone-H3 (H3K9me2) antibody or an anti-HP1 β (HP1 β) antibody as described in Chapter 3. The immunoprecipitated DNA fragments were then amplified using real-time quantitative-PCR (RTQ-PCR) using primers specifically targeting the MIEP. This new method of analysing DNA levels was developed during the course this thesis and, due to the non-chronological presentation of this data, will be explained in full in Chapter 6. Nevertheless, RTQ-PCR provides a fully quantitative analysis of the output of immunoprecipitated DNA from a ChIP assay in real-time and provides a much faster method of analysis. After quantification, low background levels of non-specific immunoprecipitation, determined by use of serum controls, was subtracted from the appropriate antibody immunoprecipitation and the data quantified relative to the input levels, taken as 10% of total. The data was then presented in chart form (Fig. 4.6).

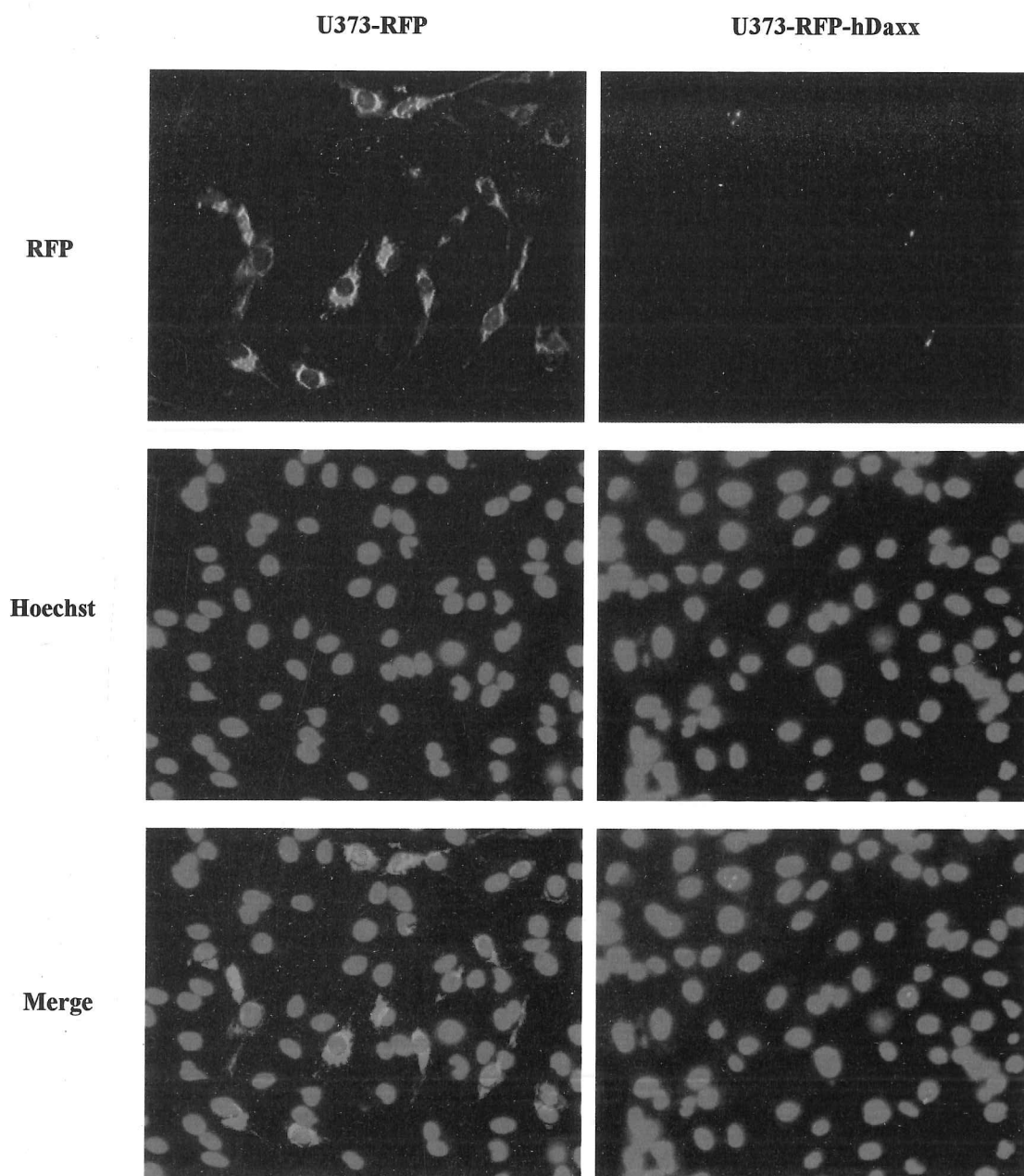


Figure 4.3 Fluorescent microscopy reveals low levels of RFP and RFP-hDaxx stably transfected U373s. U373s, either stably transfected with pDsRed-N1 (U373-RFP) or pDsRed-N1-hDaxx (U373-RFP-hDaxx), were stained with Hoechst 33342 (blue) to identify cell nuclei and then viewed under a UV fluorescent microscope for expression of RFP and RFP-hDaxx (red) respectively.

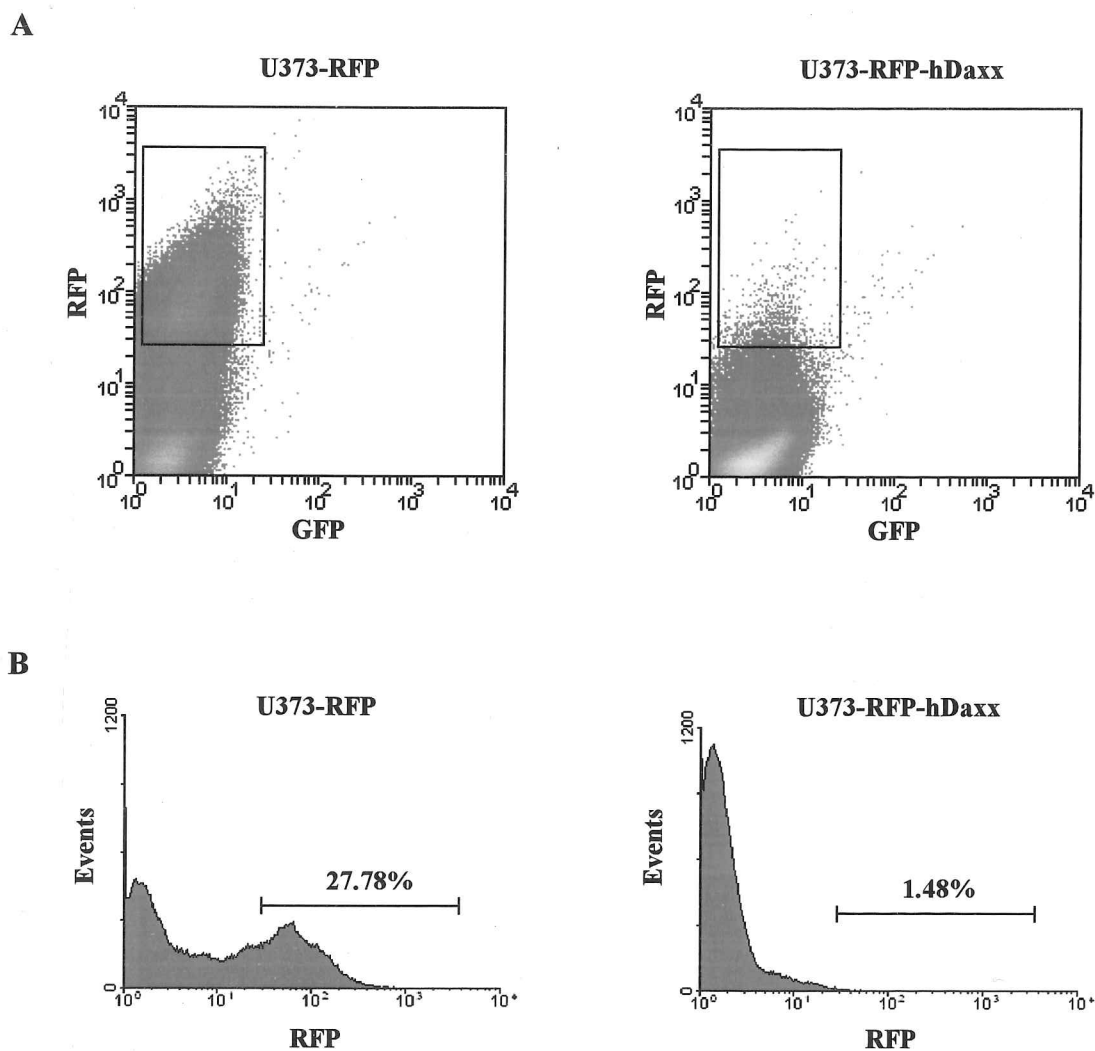


Figure 4.4 Fluorescence activated cell sorting profiles of RFP and RFP-hDaxx stably transfected U373 populations. (A) U373s, either stably transfected with pDsRed-N1 (U373-RFP) or pDsRed-N1-hDaxx (U373-RFP-hDaxx), were trypsinised from monolayers and washed in PBS before sorting of cells for RFP expression. RFP-positive cells selected to concentrate the population are shown in the open box for each. (B) A histogram of a proportion of the total cells showing the percentage of positive cells within the population.

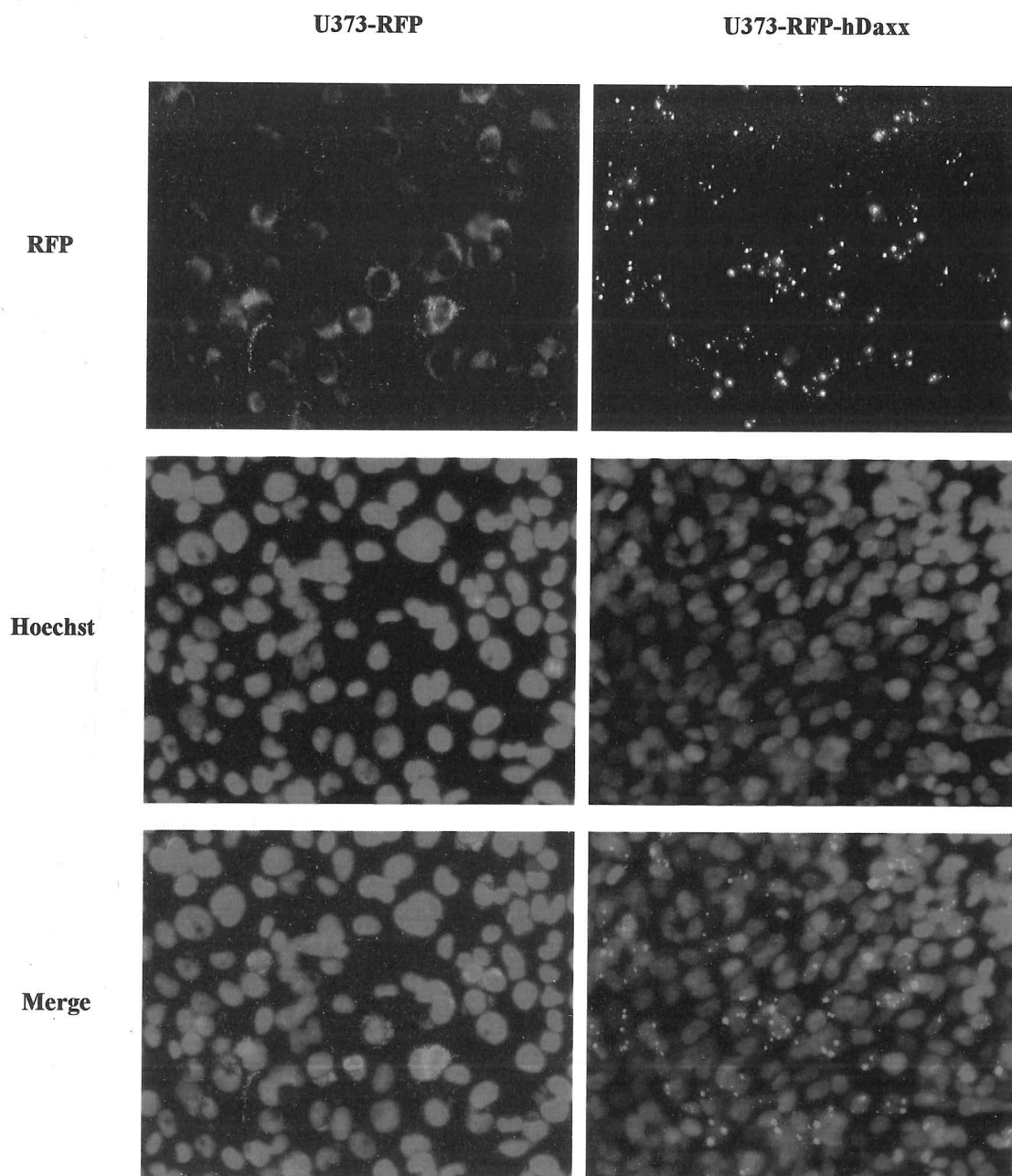


Figure 4.5 Fluorescent microscopy reveals increased levels of RFP and RFP-hDaxx stably transfected U373s after FACS. U373s, either stably transfected with pDsRed-N1 (U373-RFP) or pDsRed-N1-hDaxx (U373-RFP-hDaxx), were stained with Hoechst 33342 (blue) to identify cell nuclei and then viewed under a UV fluorescent microscope for expression of RFP and RFP-hDaxx (red) respectively.

The viral MIEP, after 24 hours of infection of the U373-RFP population, was predominantly associated with acetylated histone-H4 (Fig 4.6a, lane 1) consistent with the expression of IE genes following infection of these permissive cells. In contrast, the levels of both H3K9me2 and HP1 β were almost 15-fold lower in these cells (Fig. 4.6a, lane 2 & 3 respectively). However, the MIEPs present in the U373-RFP-hDaxx population demonstrated particularly low levels of associated acetylated histone-H4 and H3K9me2 (Fig. 4.6b, lanes 4 & 5 respectively). Similarly, the MIEP in these cells was substantially associated with HP1 β (Fig. 4.6b, lane 6). Both of these observations are consistent with the repression of IE gene expression in hDaxx over-expressing cells.

Controls to ensure consistent immunoprecipitation with each antibody were also included in these assays. Routinely, GAPDH and γ -globin promoters for either transcriptionally active (acetylated histone-H4) or transcriptionally repressed chromatin (H3K9me2 & HP1 β) were used, respectively. (The choice of promoters for each control is further explained in Chapter 6). The use of RTQ-PCR to analyse the level of acetylated histone-H4 on the GAPDH promoter showed equivalent levels of acetylated H4 for both the U373-RFP population (Fig. 4.7a, lane 1) and the U373-RFP-hDaxx population (Fig. 4.7a, lane 2). This was the same for levels of H3K9me2 and HP1 β associated with the γ -globin promoter in both populations of cells (Fig. 4.7b & c, lanes 1 & 2). Thus, with controls to show equivalent efficiency of immunoprecipitation with all antibodies, the association of the MIEP with histone markers of transcriptional activation or repression reflected the level of IE transcription in the U373-RFP and U373-RFP-hDaxx cell populations.

4.2.3 *hDaxx is transiently degraded during HCMV infection*

Clearly, hDaxx does mediate repression of IE gene expression and this repression can be relieved with the use of HDAC inhibitors. However, a recent report has suggested that HCMV infection results in the degradation of hDaxx through interaction with pp71 in a proteasomal dependent manner (Saffert & Kalejta, 2006). It was decided to confirm these observations during HCMV infection of fibroblasts (Fig. 4.8). Cells were infected with gradient purified HCMV Toledo at an MOI of 3, to infect all cells, and total cell protein was harvested from 3-12 hours post-infection (Fig. 4.8, lanes 2-5) and from control uninfected fibroblasts (Fig. 4.8, lane 1). Samples were then analysed by Western blot for IE72, hDaxx and GAPDH expression. The presence of IE72 was found by 6 hours post-

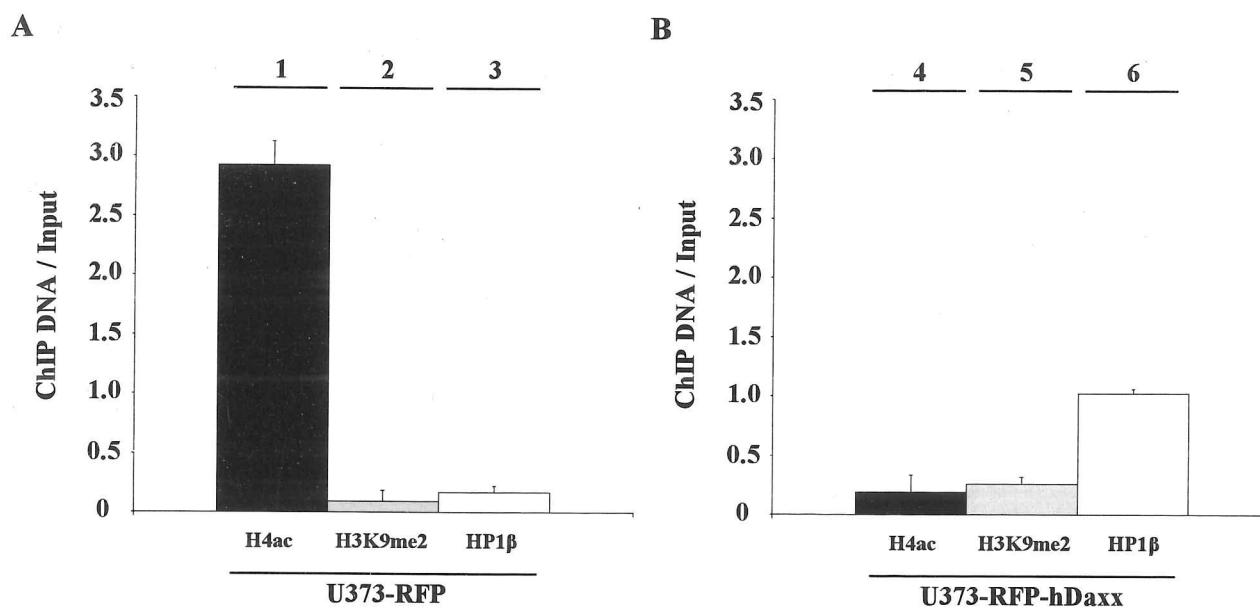


Figure 4.6 Stable expression of hDaxx in normally permissive U373 cells causes repressive chromatin at the MIEP. ChIP assays were performed (MOI 0.1) on (A) U373-RFP and (B) U373-RFP-hDaxx cells at 24 hours post-infection. Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lanes 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lanes 2 & 5) or an anti-HP1β antibody (HP1β, lanes 3 & 6). Isolated DNA was amplified using an MIEP-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

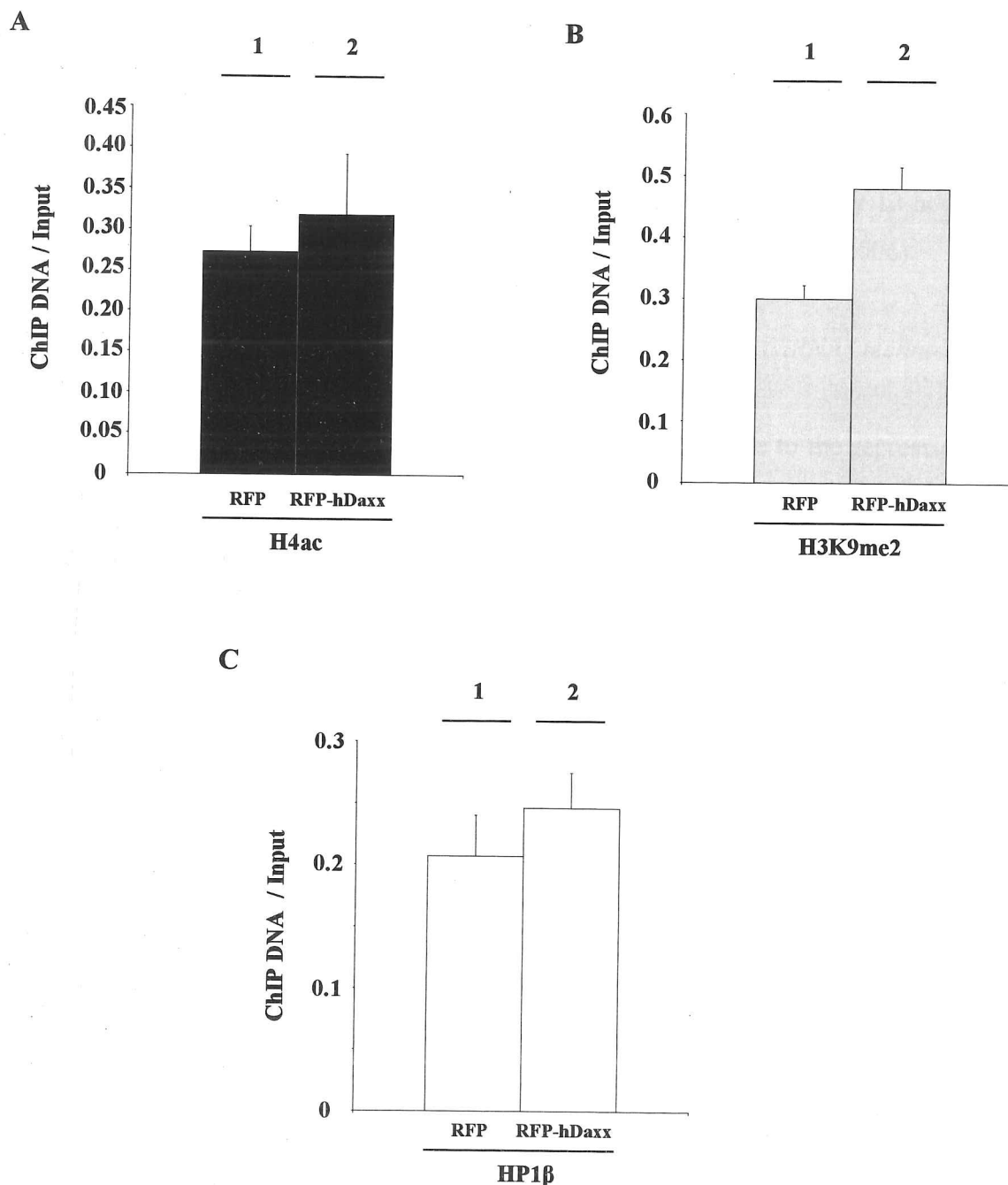


Figure 4.7 Equivalent chromatin is immunoprecipitated from U373-RFP and U373-RFP-hDaxx cells. ChIP assays were performed (MOI 0.1) on U373-RFP (lane 1) and U373-RFP-hDaxx (lane 2) cells at 24 hours post-infection. Analysis was carried out with either (A) an anti-acetylated histone-H4 antibody, (B) an anti-dimethyl lysine 9 histone-H3 antibody or (C) an anti-HP1 β antibody. Isolated DNA was amplified using either (A) GAPDH promoter-specific or (B & C) γ -globin promoter-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

infection (Fig. 4.8, lane 3) and expression continued to rise through to 12 hours post-infection (Fig. 4.8, lanes 4-5). However, by 3 hours post-infection it could clearly be seen that the level of hDaxx had reduced (Fig. 4.8, lane 2) compared to the uninfected control (Fig. 4.8, lane 1). This reduction was maximal between 3 and 6 hours post-infection (Fig. 4.8, lanes 2 & 3) before expression of hDaxx returned to normal levels by 12 hours post-infection (Fig. 4.8, lane 5). This reflected the results of Saffert & Kalejta (2006).

4.2.4 hDaxx can be down-regulated using small interfering RNA (siRNA) technology and leads to increases in HCMV IE gene expression in fibroblasts

Since hDaxx clearly contributed in the U373-RFP-hDaxx cell line to the repression of IE gene expression by chromatinisation of the MIEP in a repressive form and as hDaxx was degraded during infection of fibroblasts, it was next investigated whether down-regulation of hDaxx had any effect on IE gene expression within permissive cells using short interfering RNA (siRNA) technology. To this end, fibroblasts were either mock transfected (Fig. 4.9, lanes 1-4), transfected with a control scramble siRNA (Fig. 4.9, lanes 5-8) or with a siRNA specifically designed to target hDaxx (Michaelson & Leder, 2003) supplemented with a second commercially available hDaxx-specific siRNA (Fig. 4.9, lanes 9-12). Protein samples were then taken from transfected cells at timepoints from 24-96 hours post-transfection and the samples subjected to Western blot analysis for hDaxx and GAPDH expression. Transfection of cells with control siRNAs appeared to have little effect on the level of hDaxx expression across the timecourse in relation to GAPDH loading (Fig. 4.9, lanes 1-4 & 5-8 respectively). However, the transfection of siRNAs specifically targeting hDaxx caused a decrease in expression after just 24 hours (Fig. 4.9, lane 9) that was maximal at 72 hours post-transfection (Fig. 4.9, lane 11). Therefore, analysis of the effect of hDaxx down-regulation was routinely carried out at 72 hours post-transfection.

hDaxx down-regulation using siRNAs was further assessed by indirect immunofluorescence (Fig. 4.10). Fibroblasts were transfected with either scramble siRNA (Fig. 4.10, left panels), the two hDaxx-specific siRNAs (sihDaxx) (Fig. 4.10, middle panels) or a PML-specific commercially available siRNA as a further control (siPML) (Fig. 4.10, right panels). After 72 hours, cells were fixed, permeabilised and stained for hDaxx (green) and PML (red), using Hoechst 33342 as counter-stain for nuclei. Cells were

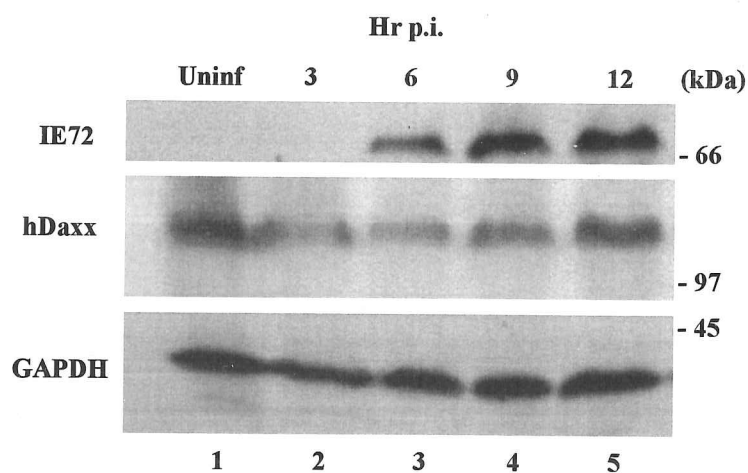


Figure 4.8 Infection with HCMV causes a decrease in hDaxx levels. Human fibroblasts were infected with gradient purified HCMV Toledo (MOI 3) and protein samples harvested between 3-12 hours (lanes 2-5) post-infection. A protein sample was also taken from uninfected cells at the time of infection (lane 1). Samples were then run on 10% polyacrylamide gels and analysed by Western blot for IE72 and hDaxx levels. GAPDH was used as a loading control.

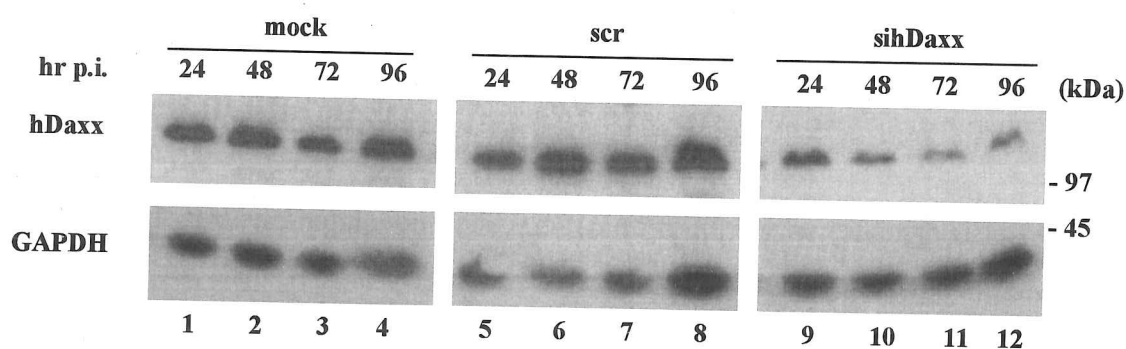


Figure 4.9 sihDaxx transfection causes transient down-regulation of hDaxx. Human fibroblasts were either mock transfected (lanes 1-4), transfected with scramble (scr; lanes 5-8) or hDaxx-specific siRNA oligonucleotide duplexes (lanes 9-12). Protein samples were harvested from 24-96 hours post-transfection and then run on 10% polyacrylamide gels before Western blot analysis for hDaxx levels. GAPDH was used as a loading control.

then viewed using fluorescent microscopy (Fig. 4.10). Scramble siRNA transfected cells showed good staining for hDaxx and PML as expected which appeared localised to ND10 (Fig. 4.10, left panels). Cells transfected with sihDaxx presented substantial down-regulation of hDaxx with no noticeable effect to PML expression (Fig. 4.10, middle panels). Interestingly, the down-regulation of PML by PML-specific siRNA also resulted in a reduced level of hDaxx staining, consistent with PML-mediated recruitment of hDaxx to ND10 (Fig. 4.10, right panels).

Since substantial down-regulation of hDaxx could routinely be established using hDaxx-specific siRNAs, the effect of hDaxx down-regulation to HCMV IE gene expression was next investigated, initially through assessment of the level of IE transcription (Fig. 4.11). Fibroblasts were mock transfected (Fig. 4.11, lane 1) or transfected with scramble (Fig. 4.11, lane 2), hDaxx-specific (Fig. 4.11, lane 3) or PML-specific (Fig. 4.11, lane 4) siRNAs. After 72 hours, the cells were infected with HCMV Toledo (MOI 0.5) and after a further 24 hours, total RNA was harvested and reverse transcription followed by PCR (RT-PCR) was carried out to determine relative RNA levels of hDaxx, PML, viral IE and GAPDH. Mock and scramble siRNA transfected cells showed similar levels of IE, hDaxx and PML RNA after 24 hours post-infection (Fig. 4.11, lanes 1 & 2). In comparison, sihDaxx transfected cells showed no change in the level of PML RNA but a decreased level of hDaxx RNA as expected (Fig. 4.11, lane 3). Interestingly, down-regulation of hDaxx resulted in a concomitant increase in IE RNA level compared to the control levels. The transfection of PML-specific siRNAs and subsequent down-regulation, seen in the PML RNA level, also caused an increase in IE RNA level, although not as considerably as with hDaxx down-regulation (Fig. 4.11, lane 4).

As down-regulation of hDaxx had led to an increase in the steady state levels of viral IE RNA, parallel analysis was next carried out to determine whether IE protein expression was similarly affected (Fig. 4.12). As before, fibroblasts were either mock transfected (Fig. 4.12, lane 1) or transfected with either scramble siRNA (Fig. 4.12, lane 2), hDaxx-specific (Fig. 4.12, lane 3) or PML-specific (Fig. 4.12, lane 4) siRNAs. After 72 hours, the cells were infected with HCMV Toledo (MOI 0.5) and after a further 24 hours, protein samples were harvested and Western blotting carried out specifically to determine relative levels of hDaxx, PML and viral major IE proteins. Coomassie staining was used as a loading

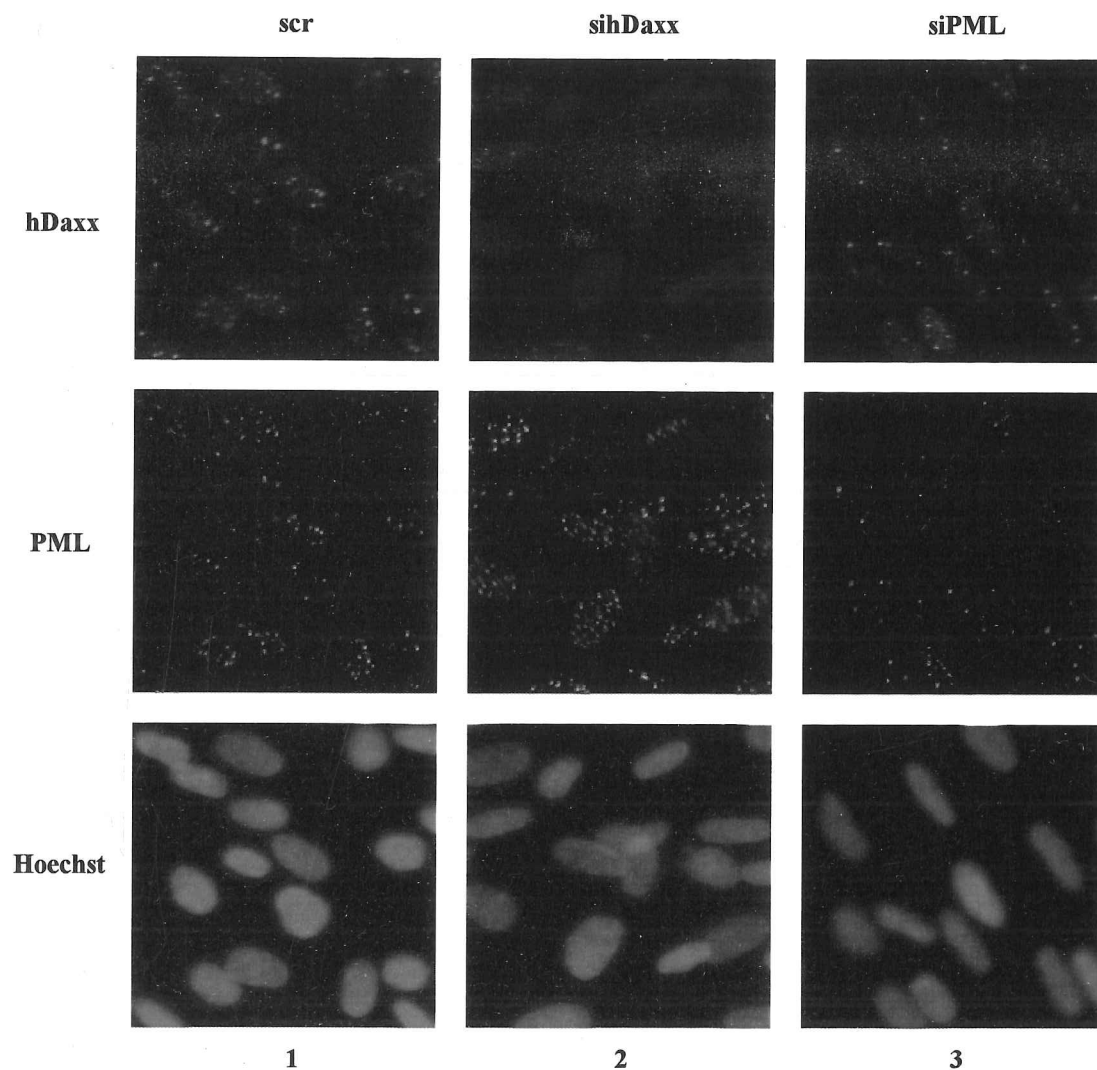


Figure 4.10 Transient down-regulation of hDaxx and PML by siRNA oligonucleotide transfection. Human fibroblasts were either transfected with scramble (lane 1), hDaxx-specific (lane 2) or PML-specific (lane 3) siRNA oligonucleotide duplexes. After 72 hours, cells were fixed and stained for hDaxx (green) or PML (red). Hoechst 33342 (blue) was used throughout to identify cell nuclei.

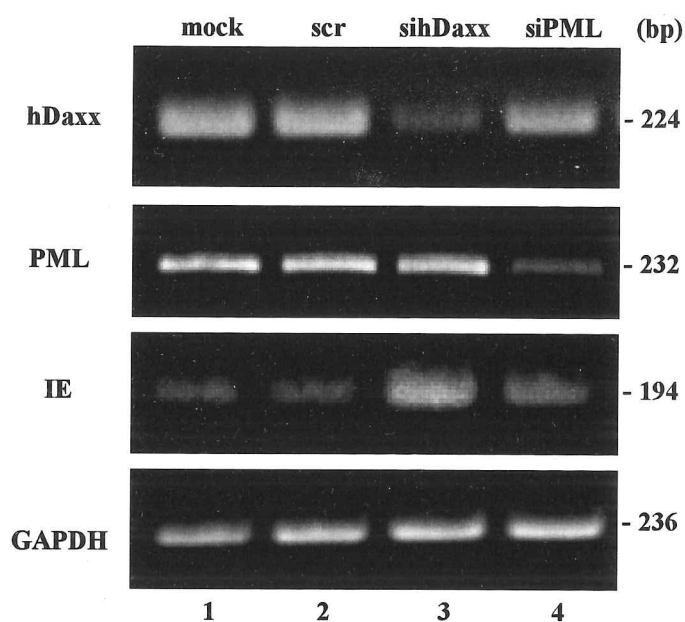


Figure 4.11 hDaxx and PML transient down-regulation causes an increase in viral IE RNA production. Human fibroblasts were either mock transfected (lane 1) or transfected with scramble (lane 2), hDaxx-specific (lane 3) or PML-specific (lane 4) oligonucleotide duplexes. After 72 hours, cells were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and extraction of total RNA. After reverse transcription, specific PCR was carried out for viral IE genes, PML and hDaxx. GAPDH was used as a loading control.

control. Mock and scramble siRNA transfected cells showed similar levels of IE72 and IE86, hDaxx and PML protein after 24 hours post-infection (Fig. 4.12, lanes 1 & 2). In comparison, sihDaxx transfected cells showed no change in the level of PML protein compared to scramble siRNA transfected cells but a decreased level of hDaxx protein, as expected, with a concomitant increase in IE72 and IE86 protein levels compared to the control lanes (Fig. 4.12, lane 3). Again, interestingly, the transfection of PML-specific siRNAs and subsequent down-regulation, seen in the PML protein level, also caused an increase in IE72 and IE86 protein levels although, as before, not to the level observed with hDaxx down-regulation (Fig. 4.12, lane 4).

Although down-regulation of hDaxx and PML had both resulted in increases in IE gene expression, it could be argued that any down-regulation of PML would automatically affect the recruitment of hDaxx to ND10. However, it was possible that the increase in IE gene expression seen with hDaxx down-regulation could have been due to non-specific effects of the siRNAs directed towards hDaxx. Thus, in order to control for possible 'off target' effects additional siRNA controls were employed, specifically an siRNA directed towards murine Daxx (mDaxx) (Fig. 4.13). Human fibroblasts and murine embryonic fibroblasts (MEFs) were either mock transfected (Fig. 4.13a & b, lane 1), or transfected with scramble siRNA (Fig. 4.13a & b, lane 2), mDaxx-specific (Fig. 4.13a & b, lane 3) or hDaxx-specific (Fig. 4.13a & b, lane 4) siRNAs. After 72 hours, the protein samples were taken from the MEF populations, whereas human fibroblast populations were infected with HCMV Toledo (MOI 0.5) and, after a further 24 hours, protein samples were harvested and Western blotting carried out to determine relative levels of hDaxx and viral major IE protein in human fibroblasts (Fig. 4.13a) and mDaxx protein in MEFs (Fig. 4.13b). Coomassie staining was used in both cases as a loading control. Mock and scramble siRNA transfected controls showed equivalent levels of hDaxx, IE72 and IE86 in human fibroblasts (Fig. 4.13a, lanes 1 & 2) and mDaxx in MEFs (Fig. 4.13b lanes 1 & 2). simDaxx transfection of human fibroblasts produced no decrease in hDaxx expression or subsequent increase in IE gene expression (Fig. 4.13a, lane 3) but down-regulated mDaxx expression in MEFs as expected (Fig. 4.13b lane 3). Additionally, sihDaxx transfection produced decreases in hDaxx expression and increases in IE gene expression as expected (Fig. 4.13a, lane 4) but had little effect on the level of mDaxx expression in MEFs (Fig. 4.13b, lane 4). All results were consistent with a robust specificity of siRNAs and previous

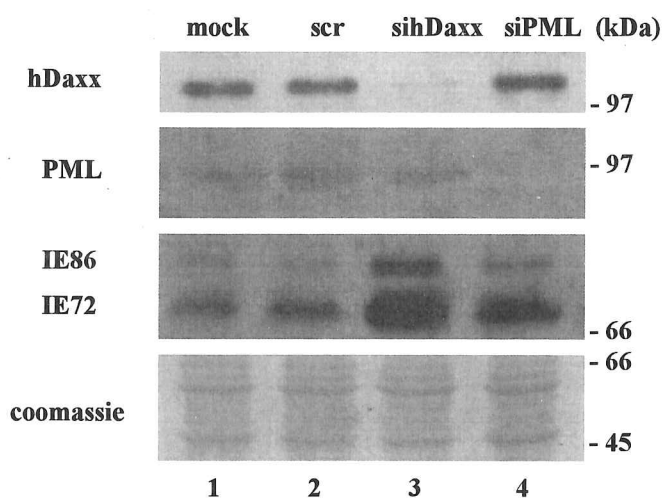


Figure 4.12 hDaxx and PML transient down-regulation causes increases in viral IE protein production. Human fibroblasts were either mock transfected (lane 1) or transfected with scramble (lane 2), hDaxx-specific (lane 3) or PML-specific (lane 4) oligonucleotide duplexes. After 72 hours, cells were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and analysis of protein content by Western blot for viral IE and hDaxx protein expression. Coomassie stained gels are presented as loading controls.

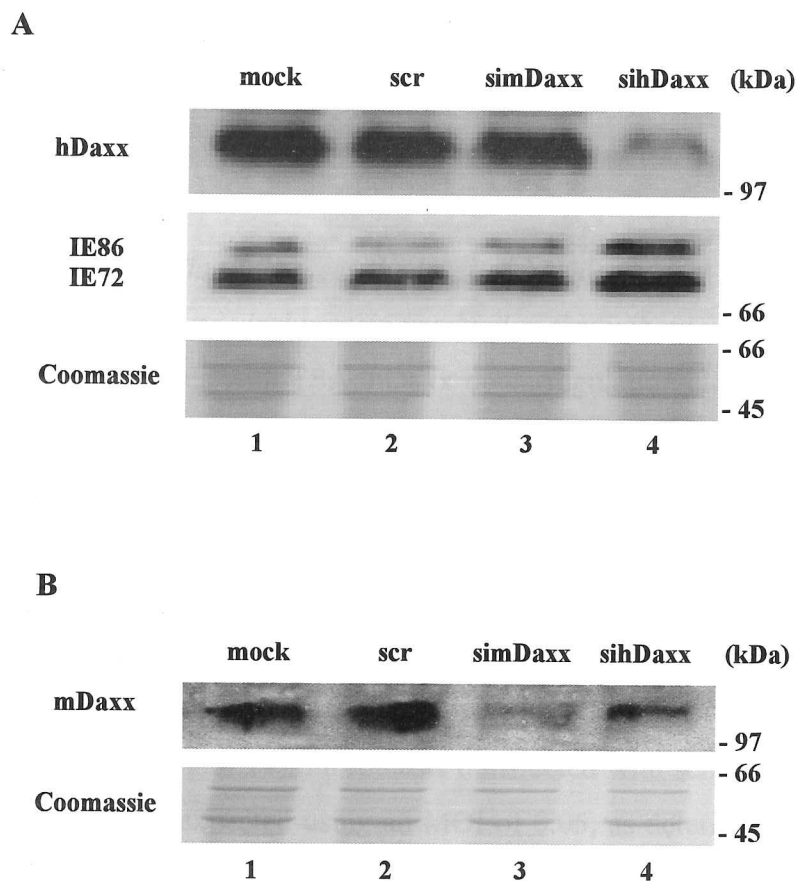


Figure 4.13 hDaxx-specific but not mDaxx-specific siRNA oligonucleotides causes increases in viral IE protein production in fibroblasts. (A) Human fibroblasts were either mock transfected (lane 1) or transfected with scramble (lane 2), mDaxx-specific (lane 3) or hDaxx-specific (lane 4) oligonucleotide duplexes. After 72 hours, cells were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and analysis of protein content by Western blot for viral IE and hDaxx protein expression. (B) MEFs were either mock transfected (lane 1) or transfected with scramble (lane 2), mDaxx-specific (lane 3) or hDaxx-specific (lane 4) oligonucleotide duplexes. After 72 hours, cells were harvested and protein content analysed by Western blot for mDaxx. Coomassie stained gels are presented as loading controls.

findings (Michaelson & Leder, 2003). Furthermore, since analysis of both RNA and protein levels had shown that scramble siRNA transfection had no profound differential effect than the mock control, a sole scramble siRNA was used when treating cells from here on in.

4.2.5 hDaxx down-regulation increases the level of permissiveness of lytic infection at low multiplicities of infection and viral protein production throughout infection

Similar to treatment of cells with TSA, hDaxx down-regulation clearly led to increases in viral IE RNA and protein levels by 24 hours post-infection. As such, it was investigated whether a concomitant increase in permissiveness of infection with HCMV also occurred. To this end, fibroblasts were transfected with either scramble siRNA (Fig. 4.14a) or hDaxx-specific siRNAs (Fig. 4.14b) for 72 hours before infection with HCMV Toledo at a low MOI (0.05). After 24 hours, cells were fixed, permeabilised and stained for IE72/86 expression with counter-staining of nuclei by Hoechst 33342 and viewed under a fluorescent microscope (Fig. 4.14). Fibroblasts transfected with scramble siRNA showed a low level of infection, as expected using an MOI of 0.05, as determined by expression of IE72/86 (green staining; Fig. 4.14a, upper panel). However, cells transfected with hDaxx-specific siRNAs showed an obvious increase in the number of cells expressing IE72/86 after infection, approximately 4-fold (Fig. 4.14b, upper panel).

As hDaxx down-regulation in HCMV infected cells had shown increases in the levels of IE gene expression and permissiveness for infection, it was next decided to determine whether hDaxx down-regulation was having subsequent effects on viral lytic infection as seen with TSA earlier (Chapter 3). Since herpesvirus gene expression is controlled in a temporally regulated cascade (Fig. 1.5), dependent upon the initial expression of in IE gene products, it was possible that increases in IE gene expression by down-regulation of hDaxx could also increase subsequent early and late transcription. Therefore, levels of early and late viral gene products, in addition to IE gene products, were assessed throughout the course of lytic infection using Western blotting after hDaxx down-regulation (Fig 4.15). Fibroblasts were transfected with either scramble siRNA or hDaxx-specific siRNAs for 72 hours before infection with HCMV Toledo at a low MOI (0.1) to aid detection of differences to the expression profiles. Protein samples were then harvested at 6, 24, 48, 72 and 96 hours post-infection and levels of expression of the major IE gene product IE72, the

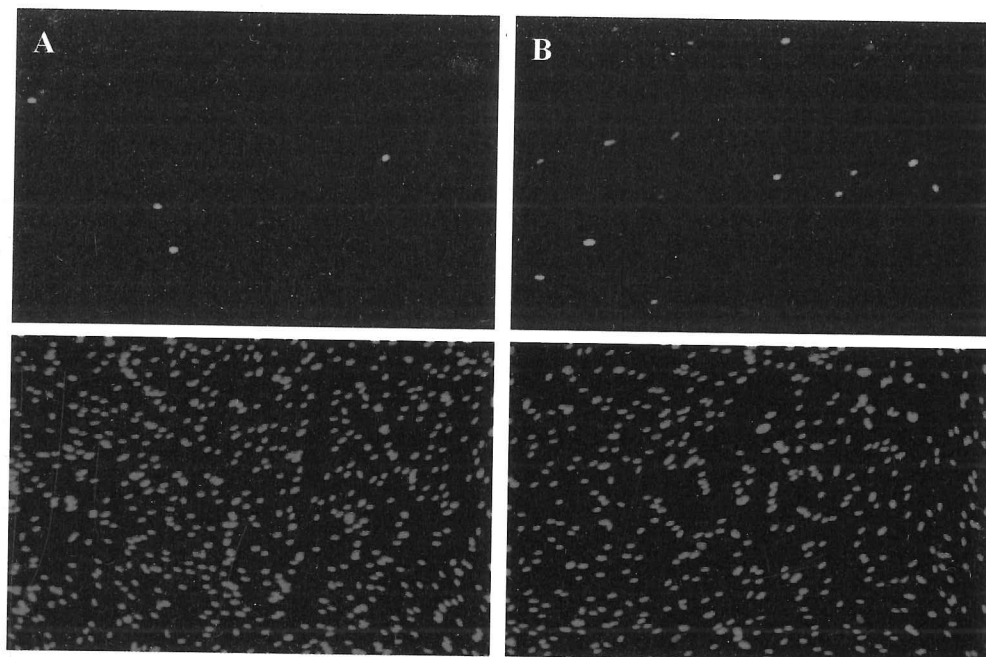


Figure 4.14 Transient down-regulation of hDaxx increases the permissiveness of infection with HCMV at low multiplicities of infection. Human fibroblasts were transfected with either (A) scramble or (B) hDaxx-specific oligonucleotide duplexes. After 72 hours, cells were infected with HCMV Toledo (MOI 0.05) for 24 hours before cells were fixed and stained for IE72/86 (green) and Hoechst 3342 (blue) and analysed by fluorescent microscopy.

early gene product UL44 and the late gene product pp28 was analysed. Low levels of IE72 were detectable in scramble siRNA transfected, infected cells by 24 hours post-infection (Fig. 4.15, lane 2) as expected, although as previously seen at this timepoint (Fig. 4.12), sihDaxx transfection resulted in an increase in the level of IE gene expression (Fig. 4.15, lane 7). Indeed, this increase in IE gene expression was observed throughout lytic infection as, in comparison to the scramble siRNA transfected cells (Fig. 4.15, lanes 3-5), the infection of sihDaxx transfected cells resulted in increased expression of IE gene products up to 96 hours post-infection (Fig. 4.15, lanes 8-10). On analysis of the early gene product, UL44, a similar temporal increase was seen in sihDaxx transfected, infected cells. Low levels of expression of UL44 in scramble siRNA transfected cells was detectable by 48 hours post-infection (Fig. 4.15, lane 3), however an increased level of expression was seen with sihDaxx transfected cells by this timepoint (Fig. 4.15, lane 8). Indeed, increased viral late gene expression was also observed in sihDaxx transfected cells: pp28 was visible by 72 hours post-infection (Fig. 4.15, lane 9) whereas in scramble siRNA transfected cells pp28 was not detected until 96 hours post-infection (Fig. 4.15, lane 5). Therefore, hDaxx down-regulation prior to infection of fibroblasts with HCMV caused an increase in the levels of gene expression of IE, early and late genes throughout the lytic cycle in already permissive cells.

4.2.6 hDaxx down-regulation causes increases in viral DNA replication and infectious virus output

hDaxx down-regulation of HCMV infected cells had clearly shown increases in the levels of IE gene expression. Consequently, the next aim was to determine whether hDaxx down-regulation had further consequences on viral lytic infection, as seen with TSA treatment of fibroblasts in Chapter 3. Fibroblasts were again transfected with either scramble siRNA or hDaxx-specific siRNAs for 72 hours prior to infection with HCMV Toledo (MOI 0.5) before DNA was extracted from cells at 6, 24, 48, 72 and 96 hours post-infection. Due to the establishment of MIEP-specific and GAPDH promoter-specific RTQ-PCR, analysis of the samples was then carried out in this manner. Levels of viral DNA are shown relative to input viral DNA quantified at 6 hours post-infection. The control scramble siRNA transfected cells (Fig. 4.16, grey bars) showed an increase in viral DNA levels by 72 hours post-infection. This increase then continued through to 96 hours post-infection, finally reaching over a 30-fold increase in the level of viral DNA compared to the input level at 6

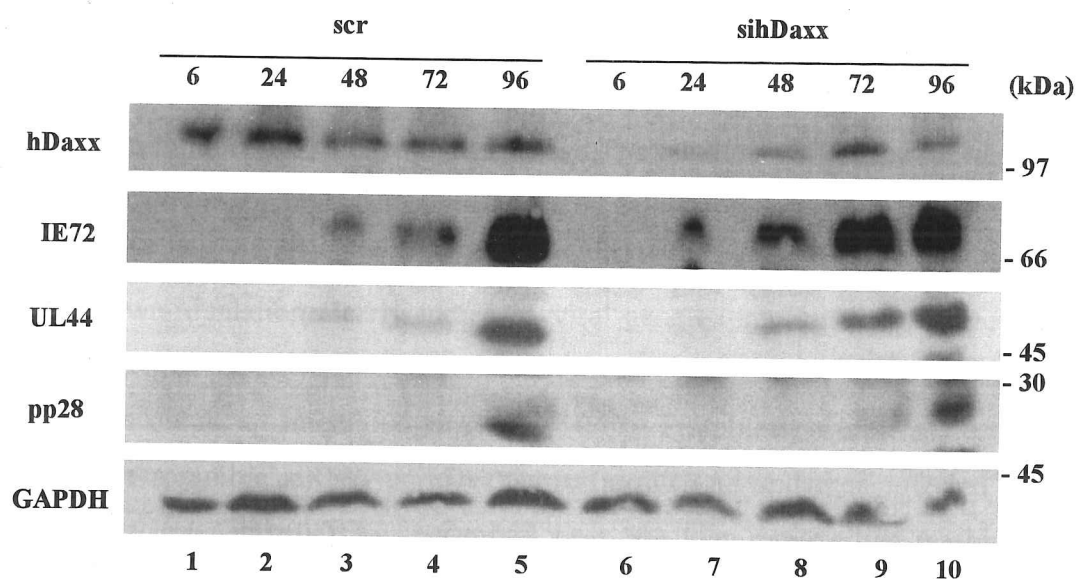


Figure 4.15 Transient down-regulation of hDaxx promotes viral protein production. Human fibroblasts were transfected with either scramble or hDaxx-specific oligonucleotide duplexes. After 72 hours, cells were infected with HCMV Toledo (MOI 0.1) and protein samples were taken at timepoints from 6–96 hours (lanes 1–5). Samples for each timepoint were run on 10% polyacrylamide gels before Western blotting for viral IE (IE72), early (UL44) and late (pp28) proteins. GAPDH was used as a loading control.

hours post-infection. In sihDaxx transfected cells, however, an increase in viral DNA levels, compared to the 6 hour input value, was detectable by 48 hours (Fig. 4.16, black bars). This increase, which was consistently above the level of viral DNA in scramble siRNA transfected cells, then continued to the 96 hour timepoint with final levels 60-fold higher than input levels. Interestingly, though, the maximal difference between the control and hDaxx down-regulated cells appeared at 72 hours post-infection.

hDaxx down-regulation clearly increased viral gene expression and DNA replication, as had TSA treatment. It was next asked, similarly, whether hDaxx down-regulation led to an increase in output of infectious virus (Fig. 4.17). Fibroblasts were transfected as before, with either scramble siRNA or hDaxx-specific siRNAs and then infected with HCMV Toledo at a low MOI (0.01). Unadsorbed virus was then removed by washing twice with fresh EMEM-10 and the medium finally being replaced. Samples of the supernatant were then taken every day for six days and the output of virus from both populations of cells assessed by plaque assay as well as the virus input used to infect both sets of cells. From day 0 to day 3 post-infection, both scramble siRNA (Fig 4.17, grey diamonds) and sihDaxx-transfected cells (Fig. 4.17, black squares) showed no infectious virus output, as expected, due to the eclipse period of the virus life cycle (Mocarski *et al.*, 2007). At 4 days post-infection, infectious virus was detectable in both control and sihDaxx transfected cells. However, by this timepoint hDaxx down-regulated cells had produced a 20% increase in the level of infectious virus progeny. The increased output from both populations of cells continued through until 6 days post-infection. However, hDaxx down-regulation resulted in 34% more infectious virus being produced by cells at this time than the scramble siRNA transfected cells.

4.2.7 hDaxx down-regulation causes changes to the chromatinisation of the MIEP at IE and early times of infection

TSA had relieved the repressive effect of hDaxx over-expression in normally permissive U373 cells. Additionally, hDaxx down-regulation in permissive fibroblasts had led to increased HCMV gene expression and replication in comparison to control cells. Furthermore, hDaxx is known to interact with HDACs and recruit these enzymes to ND10. Therefore, it was hypothesised that the repressive effect of hDaxx on the viral MIEP could be mediated through regulation of the chromatin structure of the promoter. Consequently,

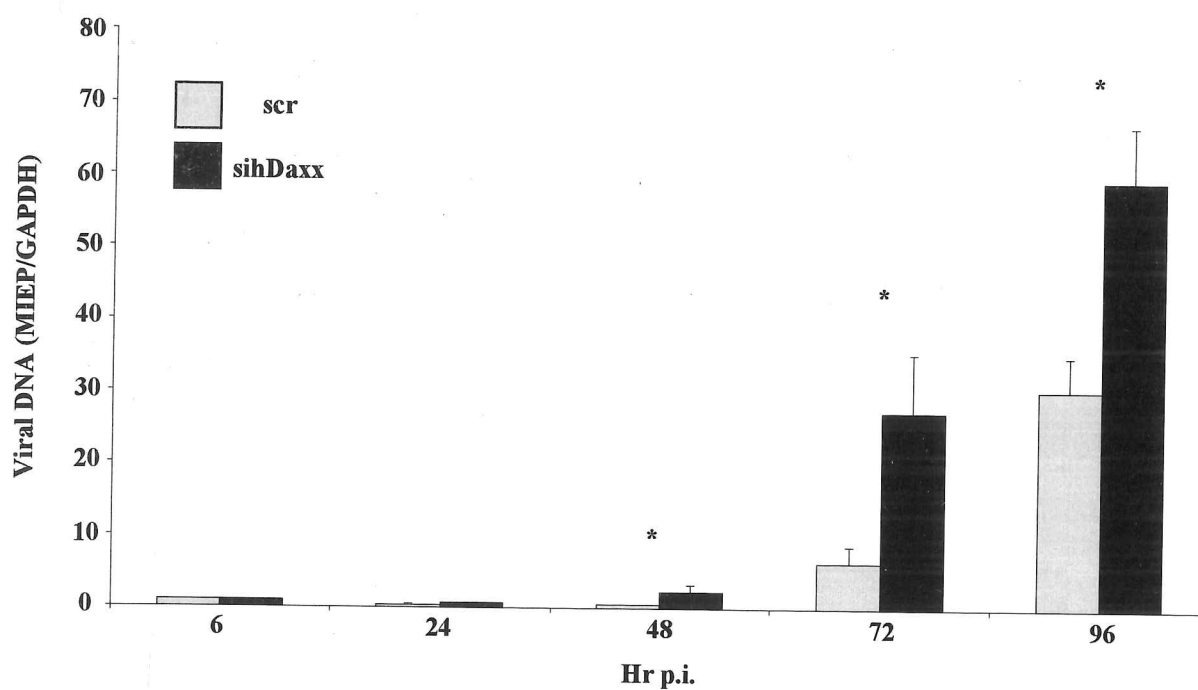


Figure 4.16 Transient down-regulation of hDaxx promotes viral DNA replication. Human fibroblasts were transfected with either scramble (grey) or hDaxx-specific (black) oligonucleotide duplexes. After 72 hours, cells were infected with HCMV Toledo (MOI 0.5) and DNA was extracted at timepoints from 6–96 hours. Both MIEP and GAPDH RTQ-PCR was then performed on samples and the quantity of viral DNA expressed as a proportion of the input DNA (MIEP/GAPDH). Error bars, 1 S.D. of three independent experiments, P value < 0.01.

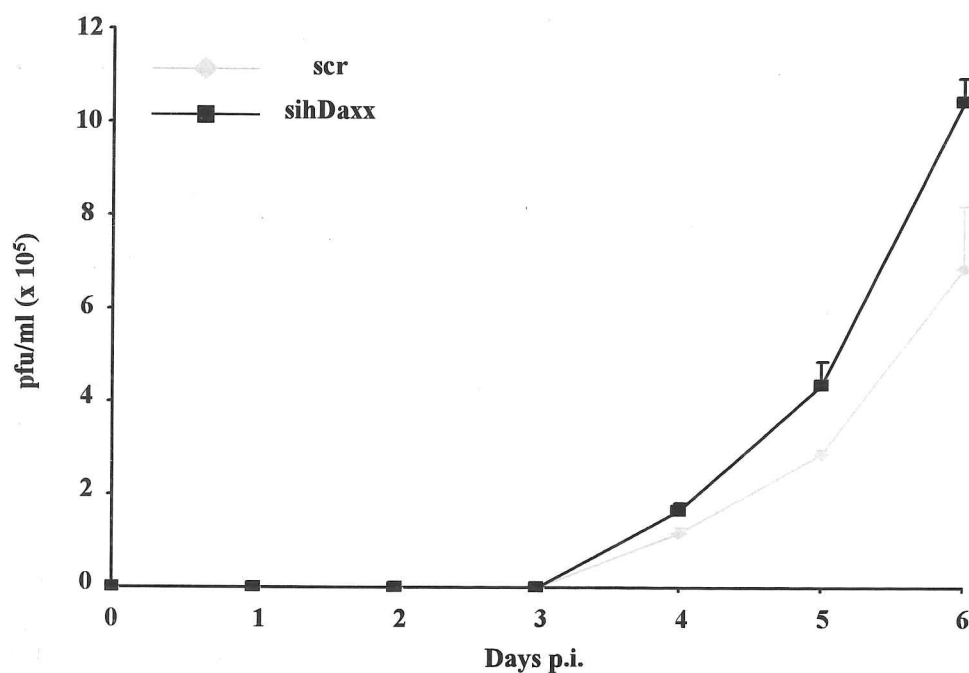


Figure 4.17 Transient down-regulation of hDaxx promotes the production of infectious virus. Human fibroblasts were treated with either scramble (grey) or hDaxx-specific (black) oligonucleotide duplexes. After 72 hours, cells were infected with HCMV Toledo (MOI 0.01) and supernatant samples were taken at timepoints for 6 days. Each sample was then used in serial dilutions to infect human fibroblasts and plaque assay analysis carried out. The data is representative of three independent experiments. *Error bars, 1 S.D.*

ChIP analyses were carried out on fibroblasts either transfected with scramble siRNA or hDaxx-specific siRNA for 72 hours to assess the viral MIEP with respect to the chromatin structure. Representative autoradiographs from the 3 hour and 24 hour post-infection samples are displayed (Fig. 4.18a & 4.20a). The intensity of amplified products from triplicate PCRs of each sample were then quantified using Image J and the data represented in chart format for both timepoints (Fig. 4.18b & 4.20b). As before, to ensure consistent immunoprecipitation of specific histone or histone associated proteins across each timepoint and throughout the full timecourse, a c-Fos PCR was carried out for both timepoints (Fig. 4.19 & 4.21).

At 3 hours post-infection with HCMV, the viral MIEP in control scramble siRNA transfected cells was predominantly associated with H3K9me2 (Fig 4.18b, lane 2), with lower but apparent levels of acetylated histone-H4 (Fig. 4.18b, lane 1). Although the level of associated HP1 β was much lower (Fig. 4.18b, lane 3) this chromatin profile is still consistent with repression immediately upon infection. In contrast, the MIEPs from sihDaxx transfected cells exhibited a very low level of association of H3K9me2 and HP1 β (Fig 4.18b, lanes 5 & 6) but a much higher level of association of acetylated histone-H4 (Fig. 4.18b, lane 4). By 24 hours post-infection, the high association of MIEPs with H3K9me2 and HP1 β in scramble siRNA transfected cells had diminished to very low levels (Fig. 4.20b, lanes 2 & 3) with much higher recruitment of acetylated histone-H4 having taken place (Fig. 4.20b, lane 1). However, in stark contrast to this, sihDaxx transfected cells now demonstrated higher levels of H3K9me2 and HP1 β (Fig. 4.20b, lanes 5 & 6) and a reduced association with acetylated histone-H4 (Fig. 4.20b, lane 4). Although no marker of repression or activation is predominant at this timepoint, this chromatin profile is consistent with transition of the promoter to a repressive structure. The control PCR for c-Fos used throughout the timecourse provided evidence that immunoprecipitation had again occurred consistently throughout the analysis of 3 hour and 24 hour post-infection samples (Fig. 4.19b & 4.21b, respectively).

4.3 Discussion

Work described in Chapter 3 showed an association of the MIEP with repressive chromatin immediately upon infection of permissive fibroblasts, likely due to HDACs. As it is known that the genomes of herpesviruses upon infection are targeted to ND10, sites of chromatin

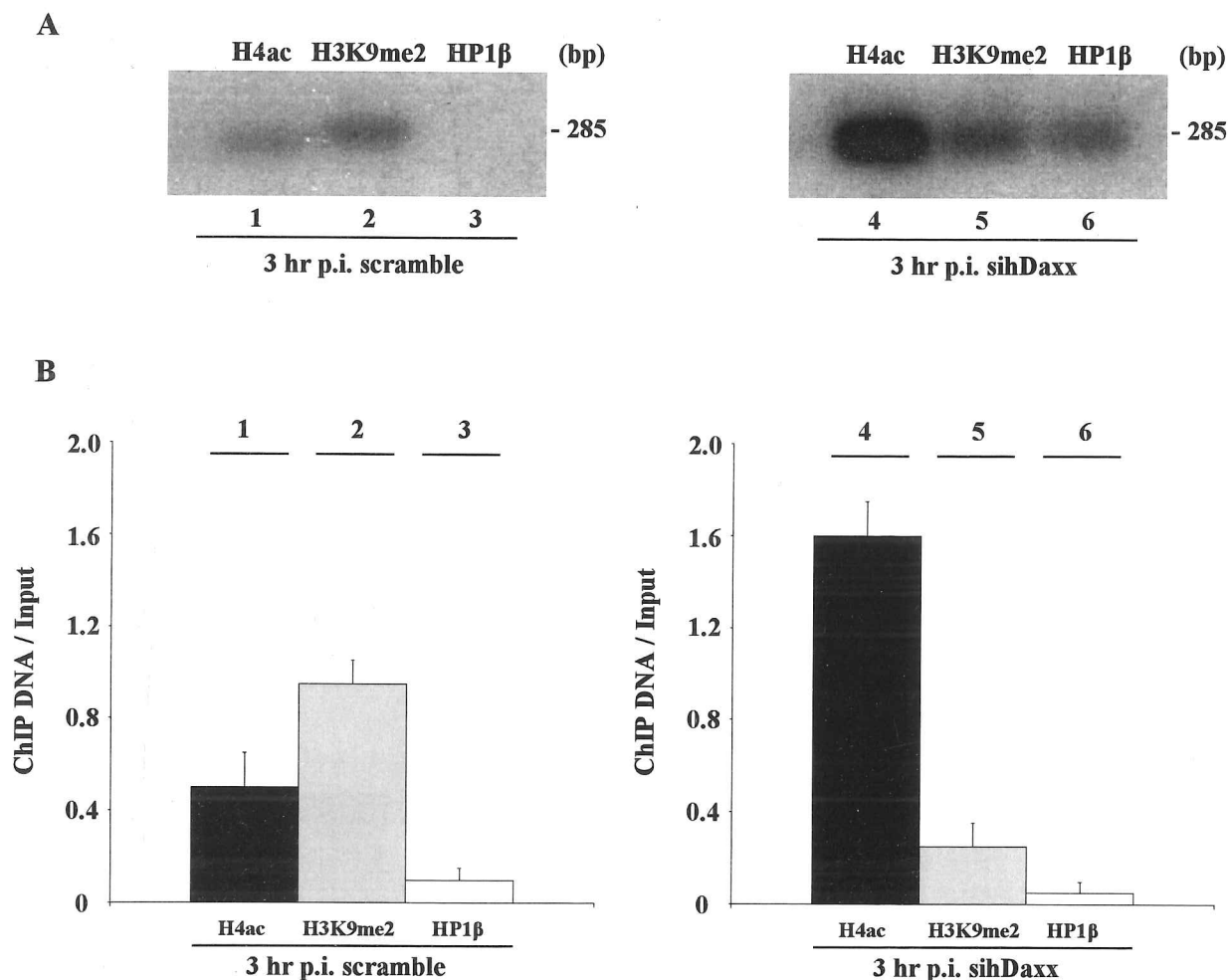


Figure 4.18 Transient down-regulation of hDaxx causes changes to the chromatinisation of the MIEP at 3 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, pre-treated with either scramble or hDaxx-specific oligonucleotides for 72 hours, at 3 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1β antibody (HP1β, lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a MIEP-specific PCR and products run on a 2% agarose gel before Southern blot analysis. (B) Image J analysis was then used to quantify autoradiograph bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

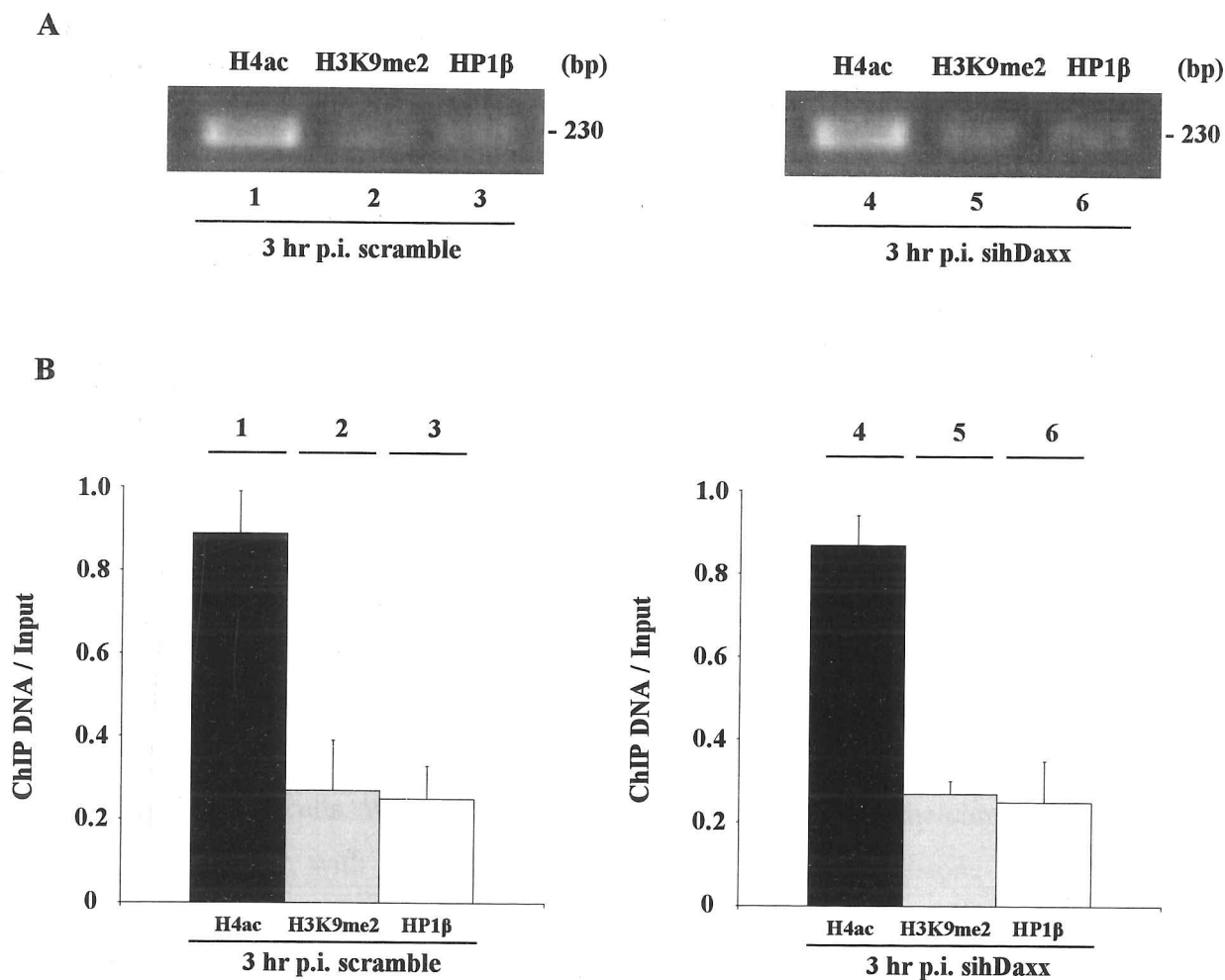


Figure 4.19 Transient down-regulation of hDaxx does not cause changes to the chromatinisation of the c-Fos promoter at 3 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, pre-treated with either scramble or hDaxx-specific oligonucleotides for 72 hours, at 3 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1β antibody (HP1β, lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a c-Fos-specific PCR and products run on a 2% agarose gel before Southern blot analysis. (B) Image J analysis was then used to quantify autoradiograph bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

remodelling enzymes, it was possible that component proteins of ND10 may be involved in mediating this repression. One such component is hDaxx which is known to interact with HDACs and had been shown previously to abrogate HCMV infection of hDaxx-stably expressing cells (Woodhall *et al.*, 2006). Therefore, the possible association of hDaxx with repression of the MIEP immediately upon infection was investigated here.

Since U373 cells stably expressing a RFP-hDaxx fusion protein (U373-RFP-hDaxx) inhibited HCMV IE gene expression, TSA was first used to investigate whether de-repression of viral gene expression was possible, as seen previously with high MOI infections (Woodhall *et al.*, 2006). Indeed, pre-treatment of U373-RFP-hDaxx cells with TSA showed over a 100-fold increase in the number of cells expressing IE protein at 24 hours post-infection. Thus, although hDaxx was still present at high levels within these cells, the use of a HDAC inhibitor was sufficient to prevent hDaxx expression from mediating its repressive effect on IE gene expression. Additionally, to support these findings ChIP analysis was carried out on the viral MIEP in control (U373-RFP) and U373-RFP-hDaxx cells. Whilst the MIEP in U373-RFP cells had the chromatin profile of an active promoter, with predominant association of acetylated histone-H4 by 24 hours post-infection, the MIEP in U373-RFP-hDaxx cells had a repressed chromatin structure, associated predominantly with HP1 β . However, the predominant association of HP1 β with the MIEP in U373-RFP-hDaxx cells does not appear as high as acetylated histone-H4 in U373-RFP cells. This may be due to the mixed population of cells assessed: only 36% of U373-RFP-hDaxx population were RFP-hDaxx positive. Therefore, some promoters may have an active chromatin structure due to their location in RFP-hDaxx negative, permissive cells. In contrast, all cells of the U373-RFP population, whether RFP-positive or not, would be permissive for IE gene expression and thus result in a clear active chromatin profile.

Other studies of the role of hDaxx in HCMV IE gene expression repression have now shown the pp71-dependent, proteasomal degradation of hDaxx upon infection. Although some have shown the complete degradation of hDaxx by pp71 by 6 hours post-infection (Saffert & Kalejta, 2006), data presented here is similar to that of other groups where hDaxx protein levels decrease from 3 hours post-infection to a maximal level by 6 hours, before re-establishing an uninfected cell level by 12 hours post-infection (Tavalai *et al.*,

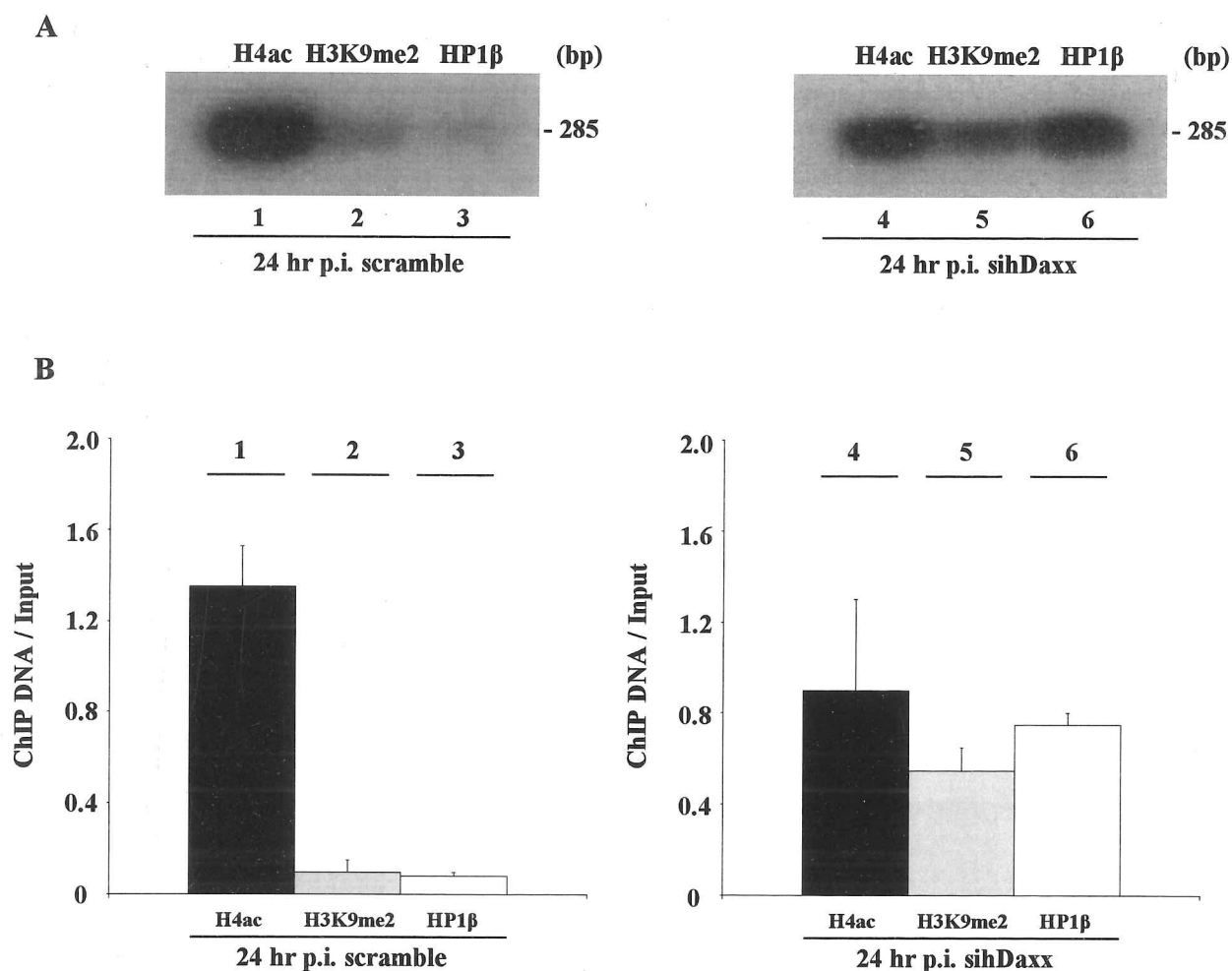


Figure 4.20 Transient down-regulation of hDaxx causes changes to the chromatinisation of the MIEP at 24 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, pre-treated with either scramble or hDaxx-specific oligonucleotides for 72 hours, at 24 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1β antibody (HP1β, lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a MIEP-specific PCR and products run on a 2% agarose gel before Southern blot analysis. (B) Image J analysis was then used to quantify autoradiograph bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

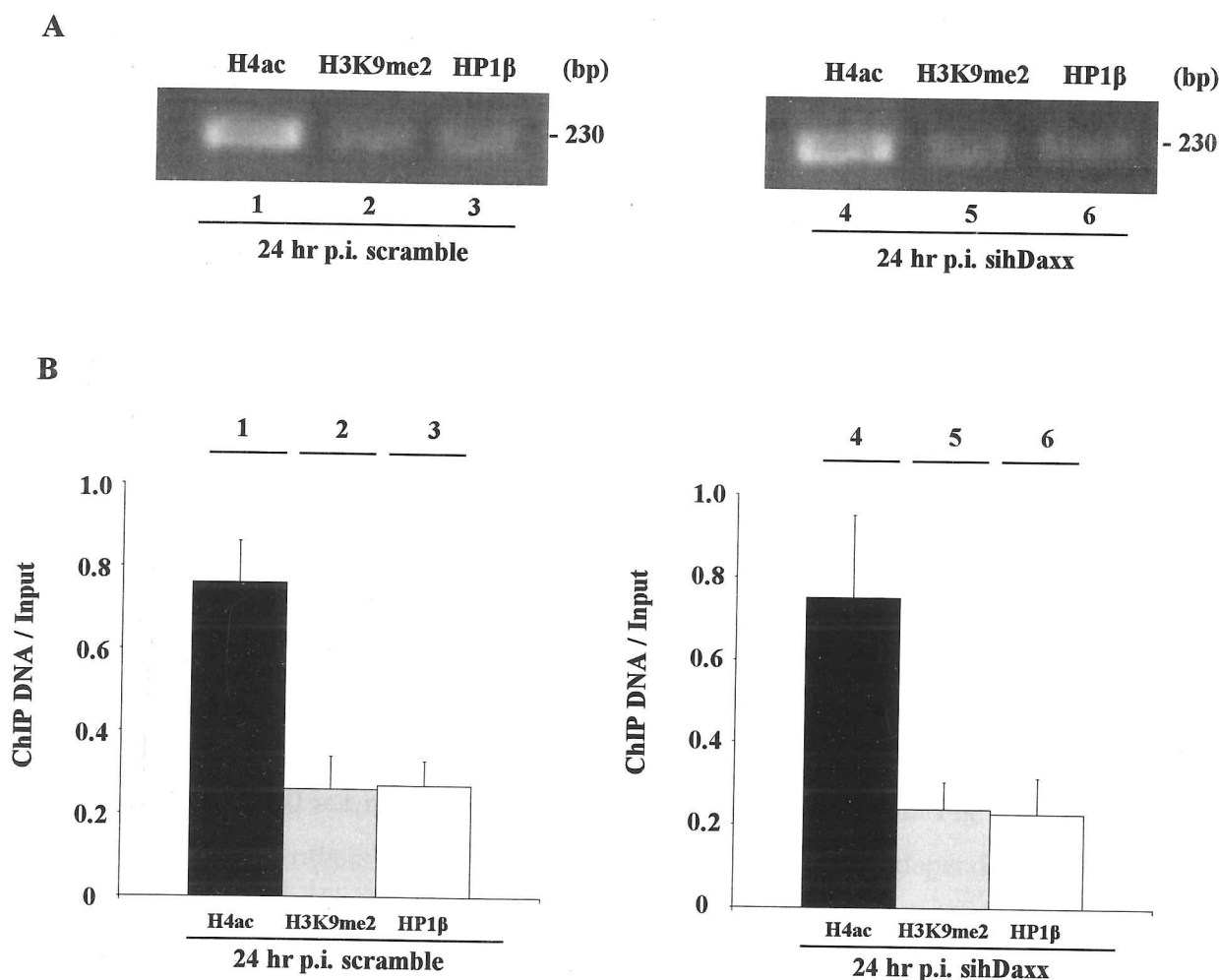


Figure 4.21 Transient down-regulation of hDaxx does not cause changes to the chromatinisation of the c-Fos promoter at 24 hours post-infection. (A) ChIP assays were performed on serum-starved human fibroblasts, pre-treated with either scramble or hDaxx-specific oligonucleotides for 72 hours, at 24 hours post-infection (MOI 0.1). Analysis was carried out with either an anti-acetylated histone-H4 antibody (H4ac, lane 1 & 4), an anti-dimethyl lysine 9 histone-H3 antibody (H3K9me2, lane 2 & 5) or an anti-HP1β antibody (HP1β, lane 3 & 6). Inputs (10% of total chromatin) are not shown. Isolated DNA was amplified using a c-Fos-specific PCR and products run on a 2% agarose gel before Southern blot analysis. (B) Image J analysis was then used to quantify autoradiograph bands in (A). For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

2008). This degradation within this time frame appears to fit with hDaxx's role as a repressor of IE gene expression which is counteracted by the tegument phosphoprotein pp71. Therefore, the use of RNA interference specifically targeting hDaxx, as previously published (Michaelson & Leder, 2003), was employed to investigate the effects of hDaxx down-regulation on viral IE gene expression. Efficient transient down-regulation of hDaxx caused an increase in the expression of IE genes by 24 hours post-infection. Such down-regulation of gene expression by RNA interference technology can be prone to off-target effects (Jackson *et al.*, 2003; Jackson *et al.*, 2006). However, although hDaxx displays 76% similarity to mDaxx across the coding region, experiments using siRNAs targeting both hDaxx and mDaxx in both human and murine fibroblasts provided no evidence for off-target effects. Interestingly, the use of a further siRNA directed towards PML, the ND10 defining component responsible for hDaxx recruitment, illustrated not only reasonable transient down-regulation of PML RNA and protein but also increases in viral IE gene expression. This result could be explained by an indirect effect of PML down-regulation on hDaxx: down-regulation of PML protein would likely affect hDaxx recruitment to ND10 but not total hDaxx protein levels (as shown in Fig. 4.10 and 4.11). However, work by others has suggested that hDaxx and PML independently contribute towards repression of IE gene expression (Tavalai *et al.*, 2006; Tavalai *et al.*, 2008) as has recently been shown for PML and Sp100 during HSV-1 infection (Everett *et al.*, 2008). PML down-regulation in subsequently infected cells is able to partly complement a pp71-deleted HCMV, although not as efficiently as hDaxx down-regulation (Tavalai *et al.*, 2008). Indeed, over-expression of PML in U373 cells has only ever been shown to delay the onset of infection but not inhibit infection (Ahn & Hayward, 2000). In contrast, hDaxx over-expression in U373 cells has shown a full abrogation of HCMV infection (Woodhall *et al.*, 2006). Hence, although PML may have some role in the regulation of HCMV IE gene expression, with PML known to interact with HDACs itself (Wu *et al.*, 2001c), repression still appears more directly linked to the greater activity of hDaxx.

Since down-regulation of hDaxx in fibroblasts had caused increases in viral IE gene expression similar to those with TSA treatment of cells, the effects of the loss of repression on full virus replication were next assessed within this system. hDaxx down-regulation increased both the permissiveness of fibroblasts for infection and the expression of all classes of HCMV genes. Similarly, the levels of viral DNA synthesis and infectious virus

output from the cells were also increased. However, increases seen with hDaxx down-regulation did not reflect the more substantial increases in viral DNA synthesis and infectious virus production with TSA treatment shown in Chapter 3. Although these results suggest a profound effect of hDaxx on the regulation of the viral MIEP, subsequent viral early and late gene expression as well as viral DNA replication, down-regulation of hDaxx alone is not sufficient to cause increases to the same level as HDAC inhibition in cells.

The substantial effect of hDaxx down-regulation on HCMV gene expression and viral DNA replication was reflected in the changes to the chromatin structure of the MIEP throughout the first 24 hours of infection. At 3 hours post-infection of scramble siRNA transfected cells the MIEP showed a predominant association with repressive markers. In contrast, in cells in which hDaxx had been down-regulated the MIEP was predominantly associated with acetylated histone-H4 consistent with the increase in viral IE gene expression within these cells. By 24 hours post-infection, the MIEP in scramble siRNA transfected cells was associated predominantly with acetylated histone-H4 as had been demonstrated at this timepoint in HCMV infected cells before (Chapter 3). However, by 24 hours post-infection, hDaxx down-regulation resulted in the MIEP becoming associated with similar levels of transcriptional markers. Although this is in contrast to the findings of TSA treated cells with HCMV infection at this timepoint, where the promoter was predominantly associated with HP1 β consistent with IE86 directed auto-regulation, the chromatin profile is consistent with a transition from an active to a repressed promoter consistent with the effects of hDaxx down-regulation on IE gene expression. In addition, these findings were fully supported by equivalent chromatin profiles at the c-Fos promoter across both timepoints.

Work using pp71 mutant viruses has shown that an interaction of pp71 with hDaxx is essential for transactivation of the MIEP and IE gene expression (Cantrell & Bresnahan, 2005). Additionally, work in which hDaxx has been over-expressed in U373 cells showed that hDaxx could inhibit IE gene expression and viral replication in a MOI dependent manner and that hDaxx knockdown using short hairpin RNA (shRNA) technology led to abolition of the impaired growth phenotype of a pp71-null HCMV mutant (Cantrell & Bresnahan, 2006). This is consistent with other experiments showing that a pp71-null HCMV could be rescued in hDaxx depleted cells (Preston & Nicholl, 2006). Additionally,

expression from the MIEP carried by a HSV-1 mutant was shown to significantly increase by 11 hours post-infection, although this could not be recapitulated during wild type HCMV infection (Preston & Nicholl, 2006). It is worthy of note though that the positive regulation by hDaxx of HCMV major IE gene expression shown previously via transfection assays and infection of Daxx-null murine cells has never been recapitulated during infection analyses with HCMV of human cells (Hoffman *et al.*, 2002; Ishov *et al.*, 2002). Indeed, consistent with data presented in this chapter, the use of siRNA technology to transiently down-regulate hDaxx expression, in comparison to a siRNA control, has been shown to increase IE72 protein expression in human fibroblasts (Saffert & Kalejta, 2006). Furthermore, the mechanism of repression of viral IE gene expression from the MIEP was also hypothesised to be mediated by HDACs as TSA treatment of cells caused increases in IE72 protein production during infection, as shown within Chapter 3 (Saffert & Kalejta, 2006).

Interestingly, it has been reported that stabilisation of hDaxx by the proteasome inhibitor lactacyctin blocks IE gene expression at low MOI (0.05) (Saffert & Kalejta, 2006). Furthermore, this proteasomal degradation of hDaxx driven by pp71 occurs in a ubiquitin-independent fashion (Hwang & Kalejta, 2007). However, other work has shown that inhibition of proteasome activity with MG132 does not completely inhibit IE gene expression and that infection of hDaxx knockdown fibroblasts treated with MG132 does not result in levels of IE gene expression observed in non-MG132 treated hDaxx knockdown fibroblasts (Kaspari *et al.*, 2008). Additionally, work presented in this chapter and by others (Tavalai *et al.*, 2008) demonstrated that although depleted by pp71, a low level of hDaxx protein remained in HCMV infected cells during IE gene expression, in contrast to data from Saffert & Kalejta (2006). Hence, proteasome inhibitors, such as MG132, appear to have other viral targets resulting in a block in late gene expression (Kaspari *et al.*, 2008).

Other viruses also appear to be inhibited by hDaxx during infection. hDaxx has been shown to interact with the viral integrase of avian sarcoma virus (ASV), early after infection of Hela cells, and mediate repression of viral gene expression, likely by recruitment of HDACs (Greger *et al.*, 2005). Additionally, hDaxx may also mediate the maintenance of repression of the virus LTR: siRNA knockdown of hDaxx, HDAC1 and

HP1 γ within ASV infected cells and TSA treatment, pp71 or IE86 expression, all lead to LTR-driven reporter gene reactivation (Poleshko *et al.*, 2008). The fas-binding domain of hDaxx has also been shown to interact with the phage Φ C31 integrase, which results in a mild loss of integration efficiency (Chen *et al.*, 2006). Hence, the repression of viral infection by hDaxx has been demonstrated at other points of a virus life cycle. Additionally, the post-translational state of hDaxx has been examined during interaction with RNA virus integrases and it has been found that an unmodified form of hDaxx acts as the repressor. This is in contrast to the likely SUMO-modified form of hDaxx found at ND10. However, it is also known that hDaxx can be post-translationally phosphorylated and that the phosphorylation status of the protein and interaction with homeodomain-interacting protein kinase 1 (HIPK1) can result in changes to hDaxx localisation and ability to repress (Ecsedy *et al.*, 2003). Hence, it is likely that these different forms of hDaxx may have independent roles within the cell as well as different effects on the virus.

Overall, it has been demonstrated that hDaxx acts as a mediator of repression of HCMV IE gene expression immediately upon infection. This most likely occurs through the recruitment of HDACs to ND10 where hDaxx interacts with the viral genome upon deposition after infection. Down-regulation of hDaxx causes the relief of repression of IE gene expression through a loss of repressive chromatin at the viral MIEP. Indeed, down-regulation is also responsible for an increase in viral gene expression throughout infection and a rise in the level of infectious viral progeny produced. However, the augmentation of HCMV infection by hDaxx down-regulation does not occur to the same level as with inhibition of HDACs. Hence, it appears that hDaxx mediates recruitment of HDACs to the MIEP which cause the repression of virus gene expression. Nevertheless, other factors able to recruit HDACs and further chromatin remodelling enzymes to ND10 may also have some role during HCMV productive infection.

5. Results

hDaxx down-regulation in *in vitro* models of latency does not permit HCMV IE gene expression

5.1 Introduction

Work presented in chapters 3 and 4, as well as work of other laboratories, has established a clear role for hDaxx in the repression of HCMV IE gene expression immediately upon infection of fully permissive cells. This repression appears to be mediated through the formation of a repressed chromatin structure at the HCMV MIEP and could be relieved by hDaxx down-regulation or HDAC inhibitors (Cantrell & Bresnahan, 2006; Preston & Nicholl, 2006; Saffert & Kalejta, 2006; Woodhall *et al.*, 2006). It is also well established that chromatin structure at the MIEP plays a major role in the maintenance of latency and reactivation of HCMV. Infection of model cell systems conditionally permissive for HCMV IE gene expression (Meier, 2001; Murphy *et al.*, 2002; Reeves *et al.*, 2005a; Reeves *et al.*, 2005b) as well as *ex vivo* culture of naturally latent CD34⁺ cells from a healthy seropositive individual (Reeves *et al.*, 2005b) has shown that the MIEP is associated with markers of transcriptional repression after infection consistent with a lack of IE gene expression. In contrast, infection of differentiated cells now permissive for IE gene expression results in association of the MIEP with transcriptionally active chromatin. Similarly, in naturally latent CD34⁺ cells, differentiation to mature DCs also results in the viral MIEP becoming associated with markers of transcriptionally active chromatin. Analogous results have been obtained using other cell lines conditionally permissive for HCMV IE gene expression (Ioudinkova *et al.*, 2006). However, it was unknown whether hDaxx had any role in the differentiation-dependent regulation of IE gene expression during virus latency or reactivation.

A number of model cell systems exist that recapitulate the differentiation-dependent regulation of viral IE gene expression observed during natural latency and reactivation of HCMV. The embryonal carcinoma cell system NT2D1 (T2 cells), which is non-permissive for viral infection due to a block in major IE gene expression, can be differentiated with retinoic acid (RA) to a fully permissive phenotype, T2RA cells (Andrews *et al.*, 1984; Gonczol *et al.*, 1984; Lubon *et al.*, 1989). Furthermore, the human acute monocytic

leukaemia cell line, known as THP1 cells, is also conditionally permissive to HCMV IE gene expression but can be differentiated to a fully permissive cell type by phorbol esters (PMA or TPA) (Weinshenker *et al.*, 1988; Sinclair *et al.*, 1992). In these cells, the differentiation-dependent regulation of major IE gene expression results from changes in the chromatin structure of the MIEP identical to those observed during natural latency and reactivation of HCMV in CD34⁺ stem cells differentiated to DCs (Murphy *et al.*, 2002; Reeves *et al.*, 2005b; Ioudinkova *et al.*, 2006). On this basis, both of these cell models have been used by a number of laboratories to mimic changes in cellular function associated with latency and reactivation of HCMV. As hDaxx had been found to mediate the chromatin based repression of HCMV IE gene expression immediately upon infection of fully permissive cells, these cell models were employed to determine whether down-regulation of hDaxx had any effect on the differentiation-dependent regulation of IE gene expression and, hence, latency and reactivation.

5.2 Results

5.2.1 Differentiation of conditionally permissive cells for HCMV infection is not associated with a decrease in endogenous hDaxx expression

It has already been established that the absolute levels of factors such as YY1 and ERF in undifferentiated T2 and THP1 cells, which are known to recruit chromatin remodelling enzymes to the MIEP and impose transcriptional repression in these undifferentiated cells, do not decrease upon differentiation (Bain *et al.*, 2003; Wright *et al.*, 2005). Instead, it is known that levels of co-factors such as HDACs are reduced (Murphy *et al.*, 2002; Wright *et al.*, 2005). As such, if hDaxx was acting as a co-repressor of viral IE gene expression in undifferentiated cells, it might be expected that endogenous levels of hDaxx would decrease upon differentiation, thus relieving this control. Therefore, the levels of endogenous hDaxx were first assessed in myeloid cells known to act as carriers of naturally latent HCMV as well as the conditionally permissive T2 cell line before and after their differentiation to cell types which would support viral IE gene expression (Fig. 5.1). Monocytes harvested from PBMC by adherence (Fig. 5.1a, lane 1) were treated with IL-4 and GM-CSF for 6 days and differentiated to immature dendritic cells (iDC) (Fig. 5.1a, lane 2), before a further 3 day treatment with LPS to mature DCs (mDC) (Fig. 5.1a, lane 3). Protein samples were harvested from each cell population and analysed by Western blot to determine the endogenous level of hDaxx; GAPDH was used as a loading control. No

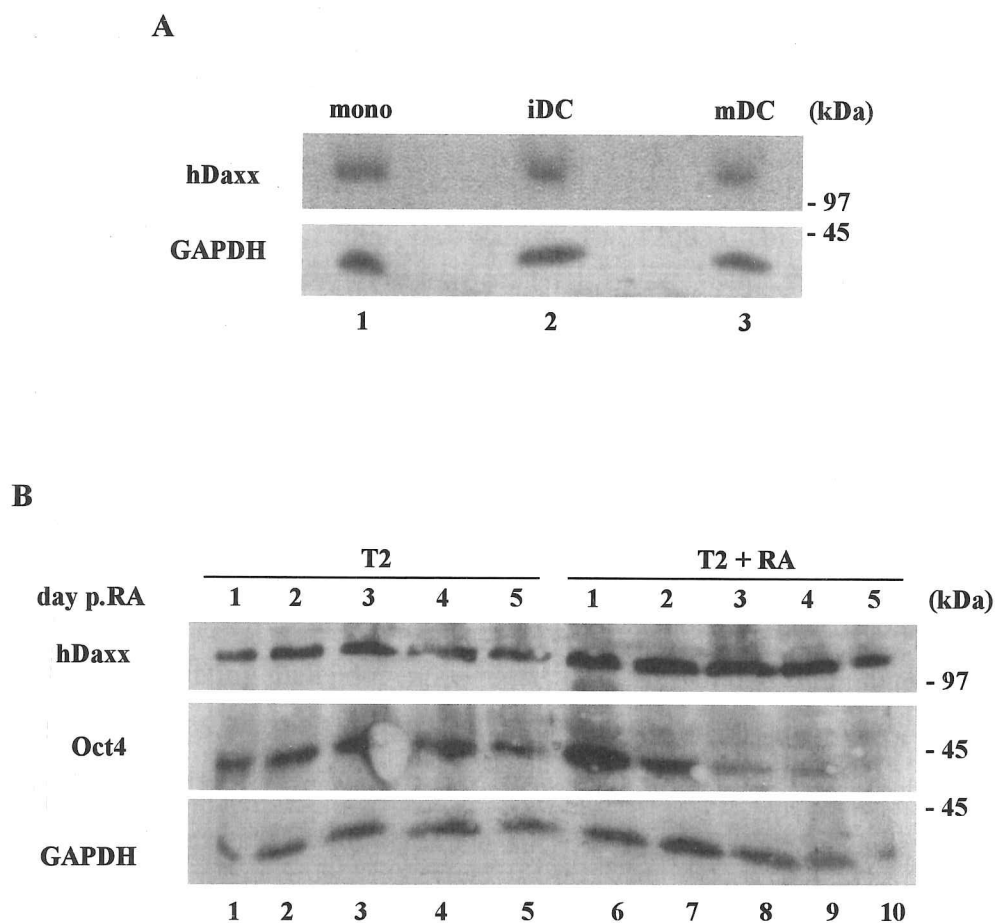


Figure 5.1 Differentiation of cells conditionally permissive to HCMV IE gene expression does not lead to a decrease in endogenous hDaxx expression. (A) Monocytes (lane 1) were treated with IL-4 and GM-CSF for 6 days and differentiated to immature dendritic cells (iDC; lane 2), before a further 3 day treatment with LPS to mature DCs (mDC; lane 3). Protein samples were harvested from each cell population and run on 10% polyacrylamide gels before Western blot analysis of hDaxx levels. GAPDH was used as a loading control. (B) T2 cells were either mock treated (fresh media; lanes 1-5) or treated with retinoic acid (lanes 6-10). Protein samples were taken from 1-5 days and run on 10% polyacrylamide gels before Western blot analysis of hDaxx expression levels. GAPDH was used as a loading control.

changes in the steady state level of hDaxx protein occurred upon differentiation of monocytes to mature DCs (Fig. 5.1a). Similarly, differentiation of T2 cells with retinoic acid for 5 days showed no change in the levels of hDaxx (Fig. 5.1b, lanes 6-10). Differentiation to T2RA cells was confirmed by the loss of Oct4 protein levels: Oct4 is a POU domain transcription factor used routinely as a marker for undifferentiated embryonic stem (ES) cells (Rosner *et al.*, 2000; Deb-Rinker *et al.*, 2005). This result is consistent with the level of RNA from T2 and T2RA cells (Fig. 5.2, lanes 1 & 4) as well as undifferentiated and differentiated THP1 cells (Fig. 5.10, lanes 1 & 4).

5.2.2 Transient down-regulation of hDaxx in T2 cells is not associated with an induction of IE gene expression or MIEP activity

As differentiation of cell lines conditionally permissive for HCMV infection due to their differentiation state showed no decrease in the endogenous level of hDaxx protein, the effect of down-regulating the endogenous levels of hDaxx in undifferentiated T2 cells was next assessed (Fig. 5.2 & 5.3a). siRNAs directed specifically to hDaxx were used as previously described in Chapter 4. T2 cells were first either mock transfected (Fig. 5.2 & 5.3a, lane 1) or transfected with scramble siRNA (Fig. 5.2 & 5.3a, lane 2) or hDaxx-specific (Fig. 5.2 & 5.3a, lane 3) siRNAs. After 72 hours, the transfected T2 cells and differentiated T2RA cells (Fig. 5.2 & 5.3a, lane 4) were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and analysis of total RNA or protein. For RNA, RT-PCR was then carried out for viral IE genes, hDaxx and Oct4; GAPDH was analysed as a loading control. Western blot analysis was employed to determine the levels of protein. The hDaxx RNA and protein level was shown to decrease substantially in sihDaxx-transfected cells (Fig. 5.2 & 5.3a, lane 3) compared to the mock and scramble siRNA controls (Fig. 5.2 & 5.3a, lanes 1 & 2). The level of reduction of hDaxx was further assessed by serial dilution of protein samples which showed that hDaxx down-regulation resulted in a decrease of over 90% of hDaxx protein (Fig. 5.3b, lanes 1-4). Further, this reduction of hDaxx had no effect on the differentiation state of T2 cells on the basis of Oct4 expression (Fig. 5.2 & 5.3a, lane 3). However, no induction of IE RNA (Fig. 5.2, lane 3) or protein (Fig. 5.3a, lane 3) occurred in these hDaxx knockdown T2 cells by 24 hours post-infection. In contrast, T2RA cells showed both an absence of Oct4 expression due to differentiation and the presence of both IE RNA (Fig. 5.2, lane 4) and protein (Fig. 5.3a, lane 4) upon infection, as expected for a permissive cell type.

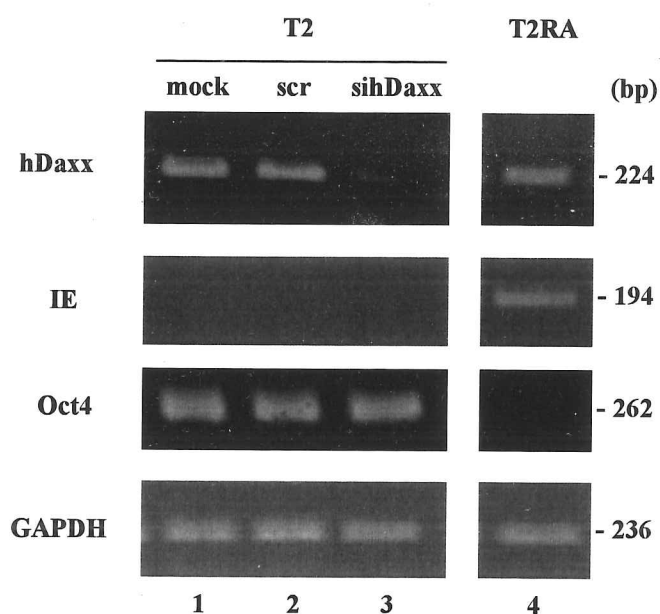


Figure 5.2 Transient down-regulation of hDaxx in T2 cells does not cause an induction of viral IE transcription upon HCMV infection. T2 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or hDaxx-specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected T2 cells and differentiated T2RA cells (lane 4) were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and extraction of total RNA. After reverse transcription, specific PCR was carried out for viral IE genes, hDaxx and Oct4. GAPDH was used as a loading control.

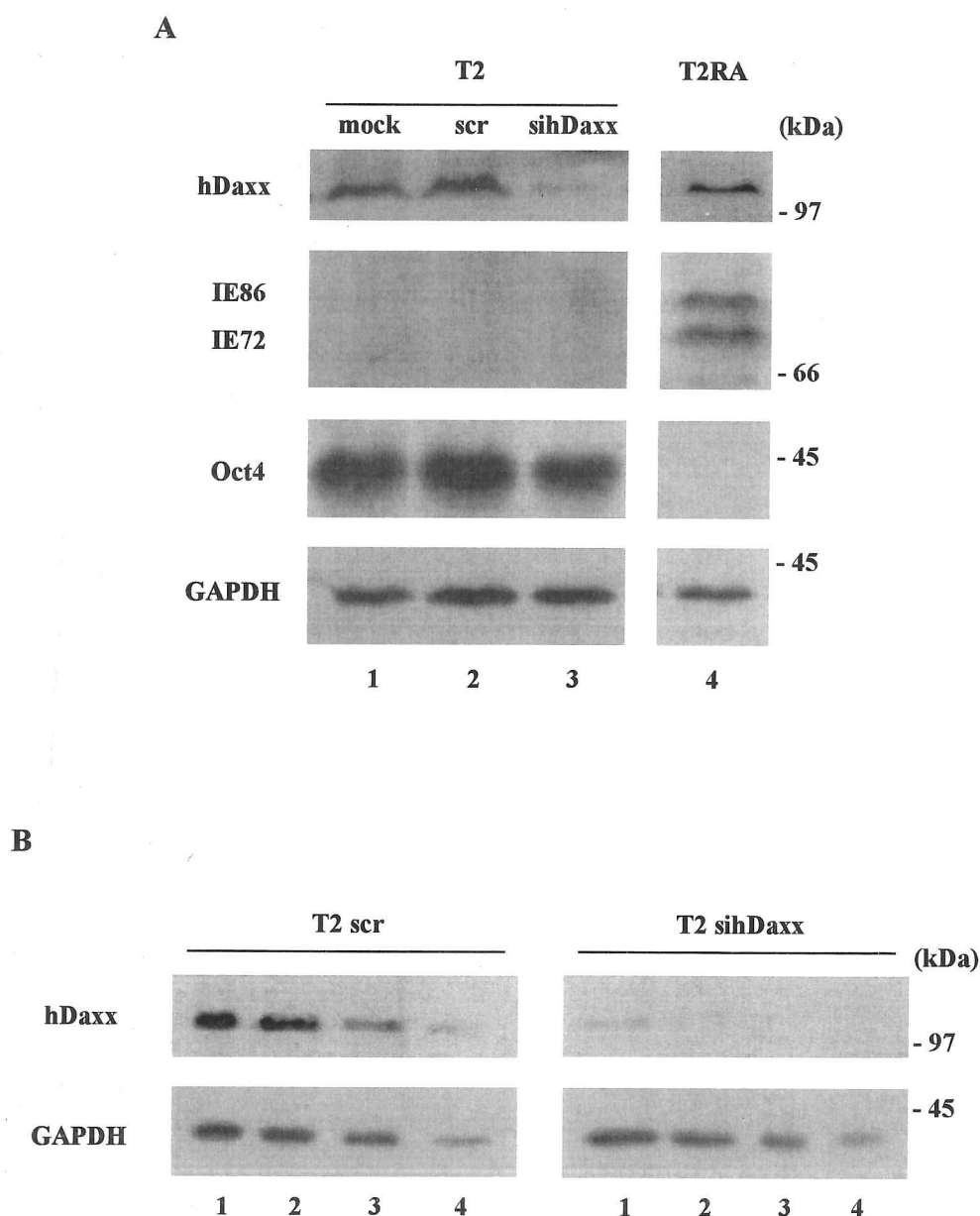


Figure 5.3 Transient down-regulation of hDaxx in T2 cells does not cause an induction of viral IE protein upon HCMV infection. (A) T2 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or hDaxx-specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected T2 cells and differentiated T2RA cells (lane 4) were infected with HCMV Toledo (MOI 0.5) for 24 hours before harvest and analysis of protein content by Western blot for viral IE, hDaxx and Oct4 protein expression. GAPDH was used as a loading control. (B) Scramble-transfected and sihDaxx-transfected T2 samples from A were re-run at 20 μ l, 12 μ l, 6 μ l and 2 μ l (lanes 1-4) per lane and Western blot analysis of hDaxx and GAPDH expression conducted.

However, the down-regulation of hDaxx in the T2 cells was not total: around 10% of hDaxx still remained in these cells. Therefore, to ensure that this residual level of hDaxx was not sufficient to maintain hDaxx-mediated repression of IE transcription, it was assessed whether the level of residual hDaxx was still able to mediate repression of a promoter known to be negatively regulated by hDaxx (Fig. 5.4). T2 cells were either transfected with scramble siRNA (Fig. 5.4a, lane 1) or hDaxx-specific siRNAs (Fig. 5.4a, lane 2). After 72 hours, transfected T2 cells were transfected with an NF- κ B-luc construct, a promoter known to be repressed by hDaxx and whose repression can be relieved by siRNAs to hDaxx (Michaelson & Leder, 2003). After a further 24 hours, cells were then harvested and NF- κ B promoter activity analysed by luciferase assay. Analysis was normalised by co-transfection with a β -gal expressing plasmid (pJATlac). The activity of the NF- κ B-luc reporter construct in sihDaxx-transfected T2 cells (Fig. 5.4a, lane 2) was increased 4.5-fold compared with scramble siRNA transfected cells (Fig. 5.4a, lane 1). In contrast, T2 cells either co-transfected with scramble siRNA (Fig. 5.4b, lane 1) or hDaxx-specific siRNAs (Fig. 5.4b, lane 2) and an interferon-stimulated response element driven luciferase construct (ISRE-luc) showed no increase in activity in sihDaxx-transfected T2 cells (Fig. 5.4b). Thus, the residual level of hDaxx observed in our transient down-regulation analysis was insufficient to maintain specific repression of a known hDaxx-repressible promoter.

In order to rule out any non-specific effects of MOI or virus strains used on the effect of hDaxx down-regulation on IE gene expression, parallel analysis of T2 cells was carried out using the highly passaged laboratory strain HCMV AD169 (Fig. 5.5). T2 cells were treated as shown in Fig. 5.3a and then infected with HCMV AD169 at an MOI of 3. Protein samples were then harvested from each cell population and analysed by Western blot for hDaxx, Oct4, GAPDH and viral IE gene expression. When GAPDH loading is taken into account, the use of a high MOI infection with a laboratory strain virus produced the same result, with hDaxx down-regulation having no effect on IE gene expression (Fig. 5.5, lane 3). Furthermore, additional analysis of the viral MIEP in transient transfection assays using a luciferase reporter construct under the control of the full length MIEP (pES-luc) showed equivalent results that hDaxx down-regulation had little to no effect on transfected MIEP activity (Fig. 5.6, lane 3) when compared to transfection controls (Fig. 5.6, lanes 1 & 2), and especially when compared to differentiated T2RA cells (Fig. 5.6, lane 4).

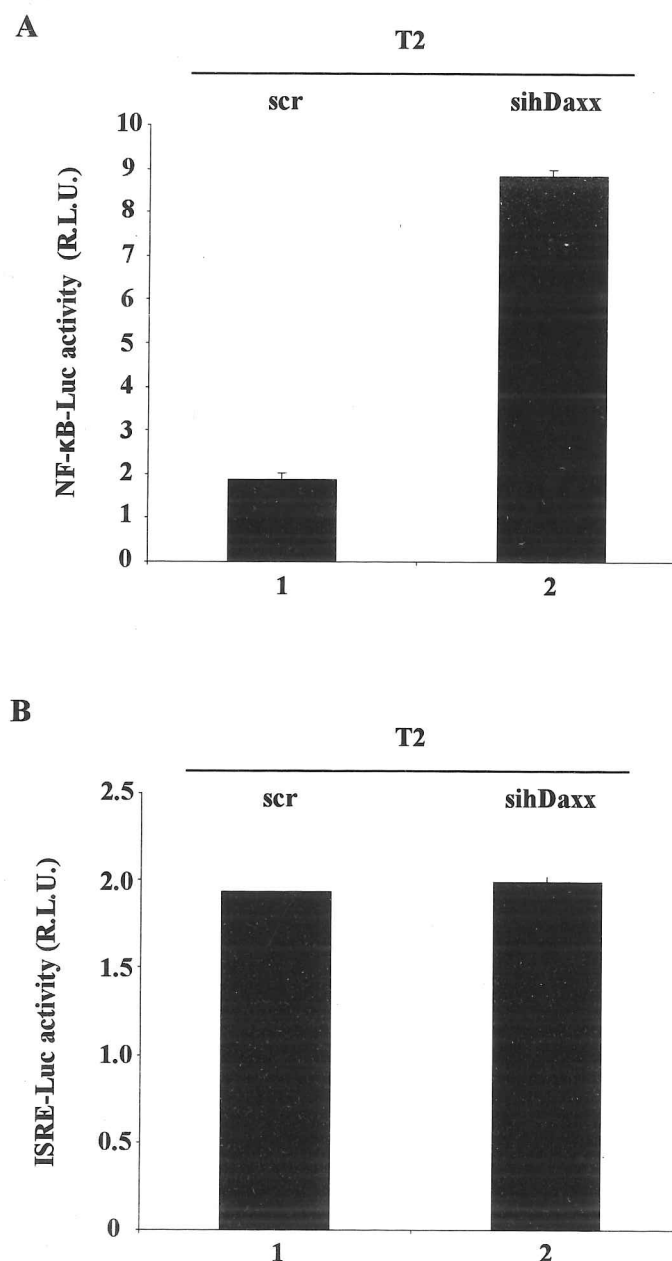


Figure 5.4 Transient down-regulation of hDaxx in T2 cells is associated with an increase in NF- κ B promoter activity. (A) T2 cells were either transfected with scramble (lane 1) or hDaxx-specific (lane 2) oligonucleotide duplexes. After 72 hours, transfected T2 cells were transfected with NF- κ B-luc. After a further 24 hours, cells were harvested and NF- κ B promoter activity analysed by luciferase assay. Analysis was normalised by co-transfection with β -gal. (B) T2 cells were either transfected with scramble (lane 1) or hDaxx-specific (lane 2) oligonucleotide duplexes. After 72 hours, transfected T2 cells were transfected with ISRE-luc. After a further 24 hours, cells were harvested and ISRE promoter activity analysed by luciferase assay. Analysis was normalised by co-transfection with β -gal. (R.L.U. = relative light units). Error bars, 1 S.D. of three independent experiments.

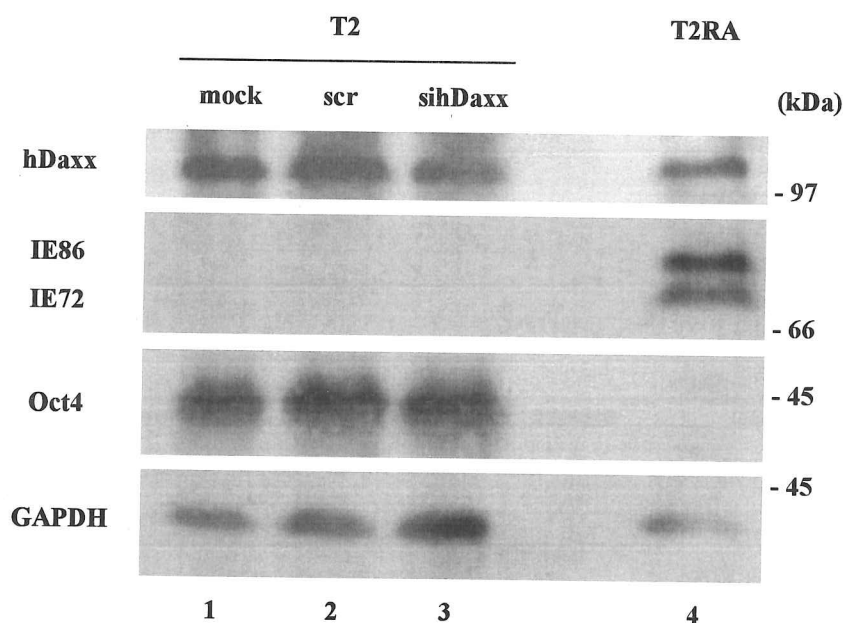


Figure 5.5 Transient down-regulation of hDaxx in T2 cells does not cause an induction of viral IE protein upon HCMV AD169 infection. T2 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or hDaxx-specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected T2 cells and differentiated T2RA cells (lane 4) were infected with HCMV AD169 (MOI 3) for 24 hours before harvest and analysis of protein content by Western blot for viral IE, hDaxx and Oct4 protein expression. GAPDH was used as a loading control.

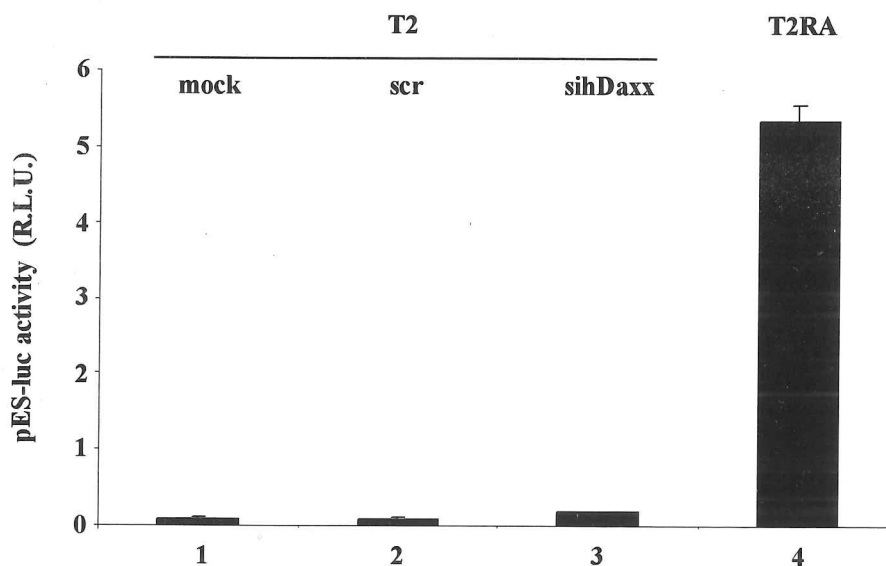


Figure 5.6 Transient down-regulation of hDaxx in T2 cells is not associated with an increase in MIEP activity. T2 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or hDaxx-specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected T2 cells and differentiated T2RA cells (lane 4) were transfected with pES-luc. After 24 hours, cells were harvested and MIEP activity analysed by luciferase assay. Analysis was normalised by co-transfection with β -gal. (R.L.U. = relative light units). Error bars, 1 S.D. of three independent experiments.

5.2.3 Expression of IE gene expression is only associated with differentiated T2 cells

The results so far had shown that down-regulation of hDaxx in undifferentiated cells did not induce IE gene expression. However, these analyses using RT-PCR, Western blot and luciferase assays were all investigations of total cell populations. Therefore, additionally, indirect immunofluorescence was employed to investigate the effects of hDaxx down-regulation on individual cells within a population (Fig. 5.7). T2 cells, transfected with either scramble siRNA (Fig. 5.7a) or hDaxx-specific siRNAs (Fig. 5.7b) for 72 hours and T2RA cells (Fig. 5.7c) were infected with HCMV Toledo (MOI 0.5). After a further 24 hours, cells were fixed, permeabilised and stained for IE protein (red) and Oct4 expression (green). Hoechst 33342 staining was used throughout for detection of nuclei. Consistent with all the analysis thus far, neither scramble siRNA transfected or hDaxx down-regulated cells showed any evidence of viral IE gene expression and Oct4 expression appeared unaffected (Fig 5.7a & b, top panels). In contrast, T2RA cells showed differentiation through loss of Oct4 staining, as expected, concomitant with high levels of permissiveness for IE gene expression (Fig. 5.7c, top panel).

5.2.4 Transient down-regulation of hDaxx in T2 cells does not affect the structure of the MIEP during infection

Since down-regulation of hDaxx in T2 cells had not resulted in any changes to IE gene expression or permissiveness of infection, the prediction would be that the MIEP would still be associated with transcriptionally repressive chromatin in these cells. Therefore, ChIP analysis was carried out to analyse this (Fig. 5.8). T2 cells transfected with either scramble siRNA (Fig. 5.8a) or hDaxx-specific siRNAs (Fig. 5.8b) as above, and T2RA cells were infected with HCMV Toledo (MOI 0.1). After 24 hours, cells were fixed and ChIP analysis carried out using either control serum, an anti-acetylated histone-H4 antibody (H4ac) or an anti-HP1 β (HP1 β) antibody. RTQ-PCR was then used to quantify association of the MIEP with these markers of transcriptional activation (acetylated histone-H4) or repression (HP1 β). Data are then presented in chart form (Fig. 5.8).

The viral MIEP, after 24 hours of infection in scramble siRNA transfected cells, was predominantly associated with HP1 β (Fig. 5.8a, lane 2), consistent with the lack of IE gene expression seen within this cell type. In comparison to this, the level of acetylated histone-H4 associated with the MIEP was between 4- to 5-fold lower (Fig. 5.8a, lane 1).

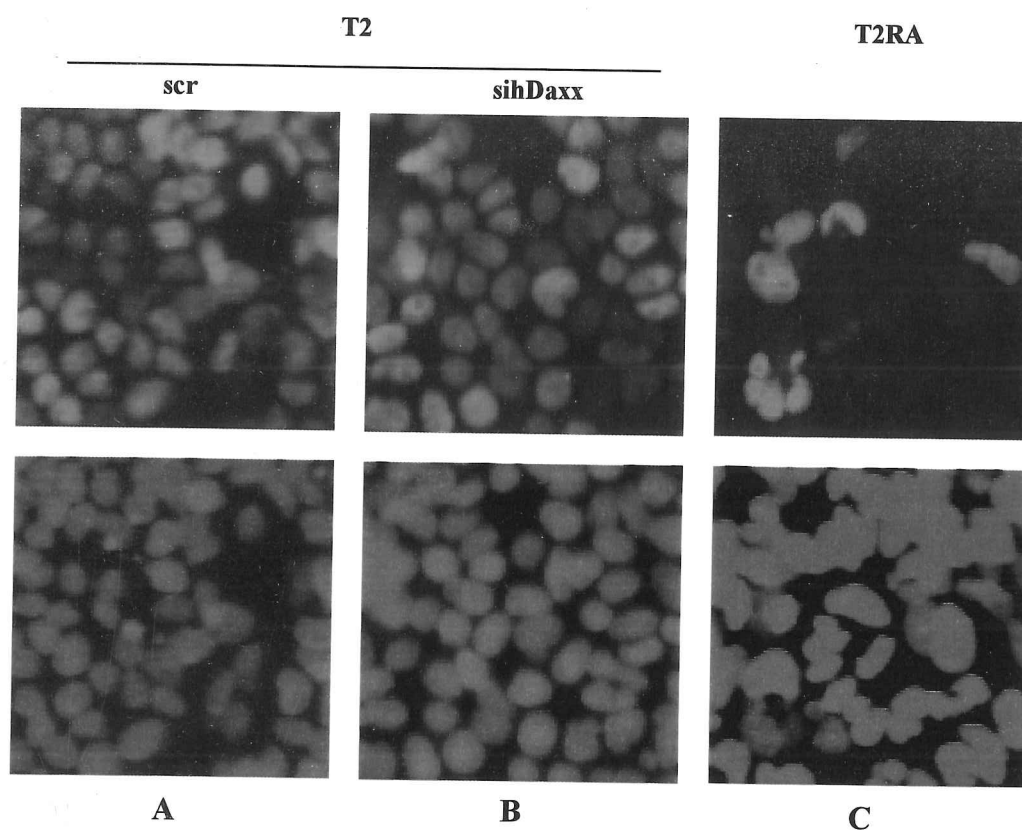


Figure 5.7 Expression of HCMV IE protein is only associated with differentiated T2RA cells. T2 cells were transfected with either (A) scramble or (B) hDaxx-specific oligonucleotide duplexes. After 72 hours, scramble and sihDaxx transfected T2 cells and (C) T2RA cells were infected with HCMV Toledo (MOI 0.5). After 24 hours, cells were fixed, permeabilised and stained for IE72/86 (red) and Oct4 (green) expression. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.

Comparison of sihDaxx-transfected T2 cells showed equivalent association of markers at the viral MIEP (Fig. 5.8b), again consistent with a lack of IE gene expression in these cells. In contrast, the MIEPs immunoprecipitated from T2RA cells demonstrated little association with HP1 β (Fig. 5.8c, lane 2) but were associated predominantly with acetylated histone-H4 (Fig. 5.8c, lane 1), as expected, with these differentiated, permissive cells (Murphy *et al.*, 2002).

As before, control RTQ-PCR using the GAPDH promoter was carried out to ensure consistent immunoprecipitation with all antibodies, with the GAPDH promoter being used since GAPDH expression did not significantly change during differentiation of T2 cells to T2RA cells (see Fig. 5.2 & 5.3a). GAPDH promoter specific RTQ-PCR showed very consistent levels of acetylated histone-H4 antibody association for all cell populations analysed (Fig. 5.9a). However, analysis of GAPDH promoters with immunoprecipitated HP1 β showed an increase in sihDaxx transfected T2 cells (Fig. 5b, lane 2) compared to scramble siRNA control transfected T2 cell (Fig. 5.9b, lane 1) with further HP1 β association upon differentiation to T2RA cells (Fig. 5.9b, lane 3). This result, however, does not confound the interpretation of analysis of the MIEP, as the level of HP1 β associated with the MIEP in hDaxx down-regulated cells is still proportional to that seen in scramble siRNA transfected cells such that the level of HP1 β associated with MIEPs in T2RA cells would be further reduced. Hence, overall, hDaxx down-regulation in undifferentiated T2 cells had little effect on the chromatin status of the promoter, whereas equivalent down-regulation in permissive fibroblasts had shown profound effects on the chromatin structure of the MIEP both at IE and early times of infection (Fig. 4.18 & 4.20).

5.2.5 Transient down-regulation of hDaxx in THP1 cells is not associated with an induction of IE gene expression

Parallel studies to those with T2 cells were carried out on conditionally permissive THP1 cell line, considered by some to be a more biologically relevant cell type (Fig. 5.10). THP1 cells were first either mock transfected (Fig. 5.10, lane 1) or transfected with scramble siRNA (Fig. 5.10, lane 2) or hDaxx-specific siRNAs (Fig. 5.10, lane 3). After 72 hours, transfected THP1 cells and differentiated THP1s (MØ) (Fig. 5.10, lane 4) were infected with HCMV Toledo (MOI 0.5) for 24 hours before either harvest and extraction of total RNA or protein. RT-PCR was then carried out for viral IE genes and hDaxx, using

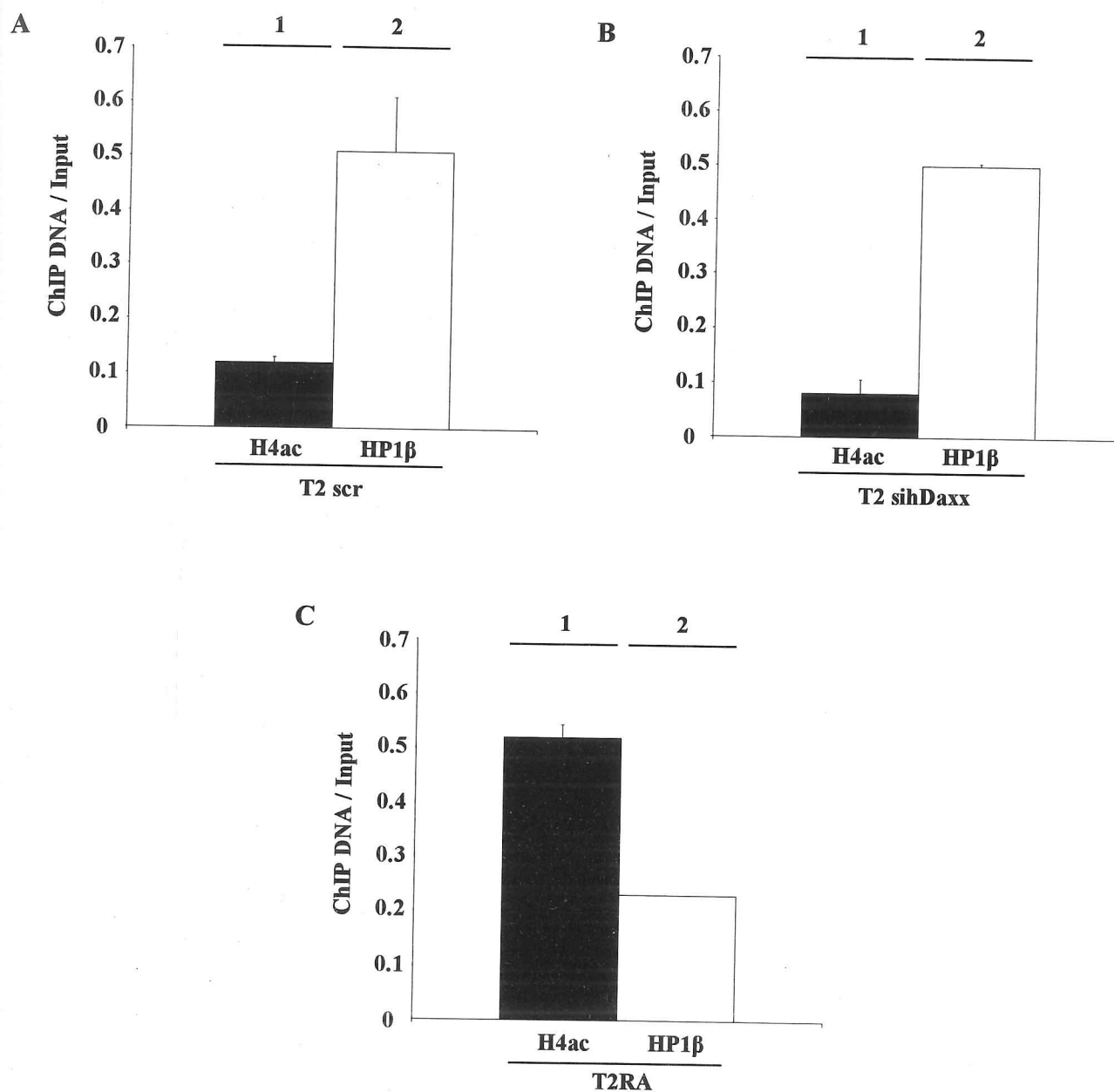


Figure 5.8 Transient down-regulation of hDaxx in T2 cells does not affect the chromatin structure of the viral MIEP and correlates with IE gene expression. ChIP assays were performed 24 hours post-infection (MOI = 0.1) on (A) scramble or (B) sihDaxx-transfected T2 cells and (C) mock-transfected T2RA cells with an anti-acetylated histone H4 antibody (lane 1) or an anti-HP1β antibody (lane 2). Isolated DNA was amplified using MIEP-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

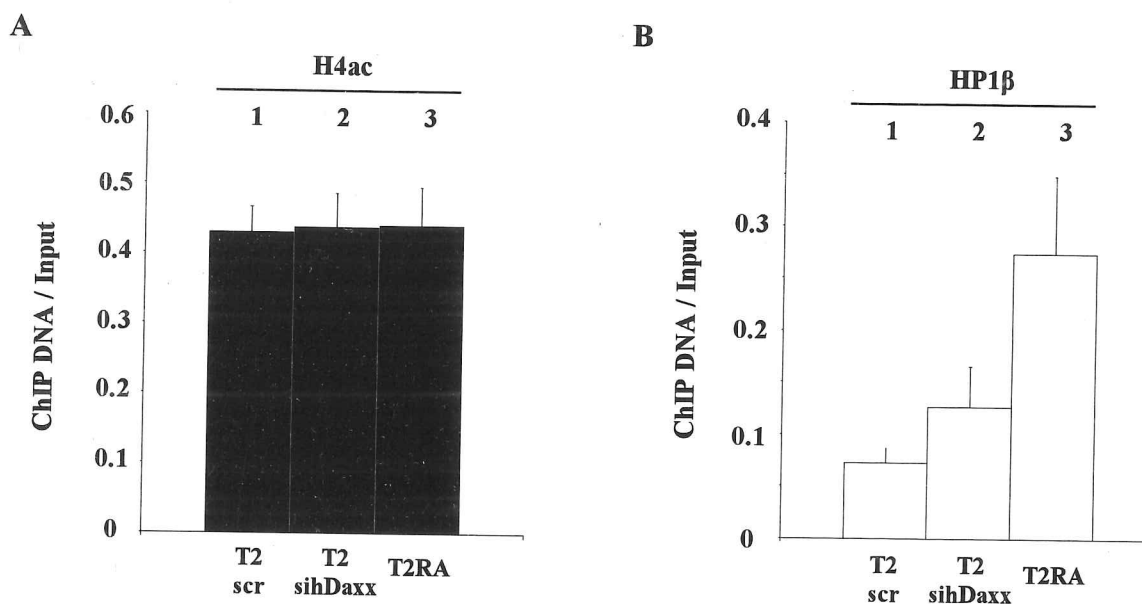


Figure 5.9 Similar levels of chromatin are immunoprecipitated from siRNA transfected T2 cells and T2RA cells. (A) ChIP assays were performed (MOI 0.1) on T2 scr (lane 1), T2 sihDaxx (lane 2) and T2RA cells at 24 hours post-infection. Analysis was carried out with an anti-acetylated histone-H4 antibody. Isolated DNA was amplified using GAPDH-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. (B) ChIP assays were performed (MOI 0.1) on T2 scr (lane 1), T2 sihDaxx (lane 2) and T2RA cells at 24 hours post-infection. Analysis was carried out with an anti-HP1β antibody. Isolated DNA was amplified using GAPDH-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

GAPDH as a loading control. Western blot analysis was also used to determine the levels of protein. The hDaxx RNA and protein level decreased substantially in sihDaxx-transfected cells (Fig. 5.10, lane 3) compared to the mock and scramble siRNA controls (Fig. 5.10, lanes 1 & 2). However, no induction of IE RNA (Fig. 5.10a, lane 3) or protein (Fig. 5.10b, lane 3) occurred in sihDaxx transfected THP1 cells by 24 hours post-infection. In contrast, differentiated THP1 cells showed good expression of both IE RNA (Fig. 5.10a, lane 4) and protein (Fig. 5.10b, lane 4) upon infection. Therefore, down-regulation of hDaxx in this cell line also had no effect on induction of IE gene expression.

5.2.6 Oct4 down-regulation in T2 cells is associated with induction of IE gene expression through differentiation

Accumulating evidence from these experiments suggested that HCMV IE gene expression could only be induced by differentiation of conditionally permissive undifferentiated cell types and not by down-regulation of hDaxx expression. In T2 cells, differentiation can be assayed by the level of down-regulation of endogenous Oct4 (POU5F1) (Rosner *et al.*, 1990; Rosfjord & Rizzino, 1994; Deb-Rinker *et al.*, 2005), a transcription factor known to be a master regulator of differentiation which is expressed in ES cells and embryonal carcinoma cells, such as T2 cells (Chambers & Smith, 2004; Hart *et al.*, 2004). As other octamer proteins were known to be involved in the regulation of herpesvirus IE gene expression (see 5.3 Discussion), it was possible that Oct4 may also have a role in repression of the MIEP in undifferentiated cells which is relieved upon differentiation of these cells to T2RA cells as loss of Oct4 expression occurs. Therefore, siRNA analysis was carried out to determine if specific down-regulation of Oct4 had any effect on HCMV IE gene expression in T2 cells.

Initially, it was first established that use of a commercially available siRNA targeting Oct4 resulted in decreased Oct4 expression in T2 cells (Fig. 5.11). Analogous to hDaxx-specific down-regulation in Chapter 4, T2 cells were either mock transfected (lane Fig. 5.11, lanes 1, 4, 7 & 10) or transfected with scramble siRNA (Fig. 5.11, lanes 2, 5, 8 & 11) or Oct4-specific siRNA (Fig. 5.11, lane 3, 6, 9 & 12). Protein samples were then harvested from 24-96 hours post-transfection and analysed by Western blot for Oct4 expression levels. GAPDH was used as a loading control. As can be clearly seen, siOct4-transfected cells showed down-regulation of Oct4 by 24 hours post-transfection (Fig. 5.11, lane 3)

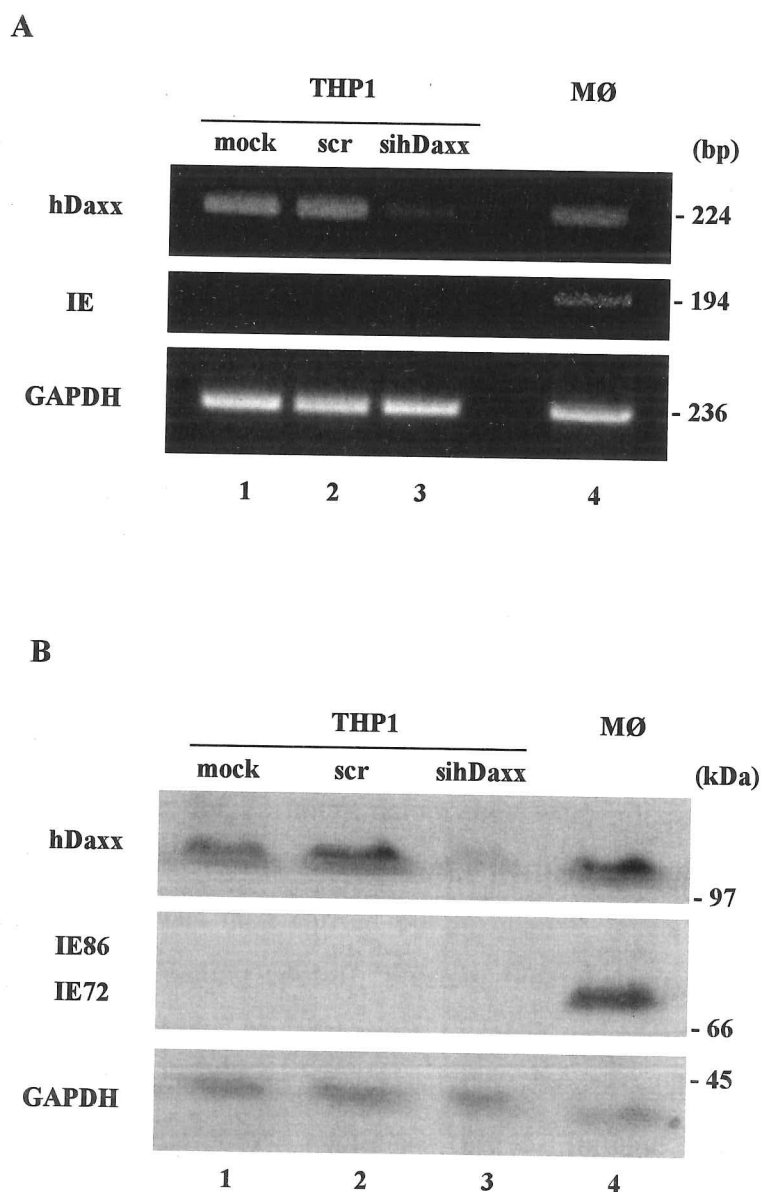


Figure 5.10 Transient down-regulation of hDaxx in THP1 cells does not cause an induction of viral IE gene expression upon HCMV infection. THP1 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or hDaxx-specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected THP1 cells and differentiated THP1 cells (MØ) (lane 4) were infected with HCMV Toledo (MOI 0.5) for 24 hours. (A) Cells were then harvested and total RNA extracted. After reverse transcription, specific PCR was carried out for viral IE genes and hDaxx. GAPDH was used as a loading control. (B) Cells were harvested and protein content analysed by Western blot for viral IE and hDaxx expression. GAPDH was used as a loading control.

compared with control transfected cells (Fig. 5.11, lanes 1 & 2). This down-regulation increased maximally by 48 hours post-transfection (Fig. 5.11, lane 6). Consequently, this protocol was used to investigate whether Oct4 down-regulation in T2 cells could cause changes to the activity of the MIEP after transfection. Luciferase assays using the full length MIEP driven luciferase construct (pES-luc) were carried out as follows: T2 cells were either mock transfected (Fig. 5.12, lane 1) or transfected with scramble siRNA (Fig. 5.12, lane 2) or Oct4-specific siRNAs (Fig. 5.12, lane 3). After 72 hours, cells including T2RA cells as a positive control for MIEP activity (Fig. 5.12, lane 4), were transfected with pES-luc. Levels of luciferase activity were again normalised to levels of expression of β -gal from a co-transfected pJATlac construct. These results showed that Oct4 down-regulation in T2 cells caused a substantial increase to MIEP activity (Fig. 5.12, lane 3), when compared to the control transfected T2 cells (Fig. 5.12, lanes 1 & 2). Indeed, the activity of the MIEP in Oct4 down-regulated T2 cells was almost equivalent to that in the differentiated T2RA cell population (Fig. 5.12, lane 4).

Since activity of the MIEP increased with down-regulation of Oct4, the effect of this decrease in Oct4 expression on virus infection was next investigated (Fig. 5.13). T2 cells were transfected, as above, for 72 hours before they were infected with HCMV Toledo (MOI 0.5) for 24 hours. T2RA cells were used as a positive control for IE gene expression after infection. RT-PCR was then carried out to analyse levels of IE gene and Oct4; GAPDH was used as a loading control. Western blot analysis was also employed to determine the levels of protein. In comparison to mock and scramble siRNA transfected controls (Fig. 5.13, lanes 1 & 2), the down-regulation of Oct4 caused induction of both IE RNA (Fig. 5.13a, lane 3) and protein expression (Fig. 5.13b, lane 3). Differentiated T2RA cells also showed IE gene expression, as expected (Fig. 5.13, lane 4). Additionally, indirect immunofluorescence was used to confirm that Oct4 down-regulation induced IE gene expression (Fig. 5.14). T2 and T2RA cells transfected and infected as above, 24 hours post-infection, were fixed, permeabilised and stained for presence of IE protein (red) and Oct4 (green) protein, with Hoechst 33342 staining used to counter-stain nuclei. In comparison to scramble siRNA transfected control cells (5.14a, upper panel), T2 cells transfected with siOct4-specific siRNAs showed clear levels of Oct4 down-regulation concomitantly with an induction of IE72/86 expression (Fig. 5.14b, upper panel). High

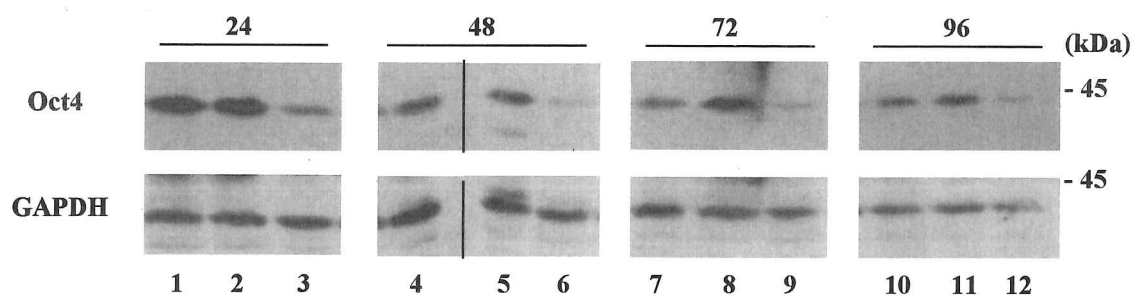


Figure 5.11 Oct4-specific siRNA oligonucleotides cause down-regulation of Oct4 in T2 cells. T2 cells were either mock transfected (lane 1), transfected with scramble (lane 2) or Oct4-specific (lane 3) siRNA oligonucleotide duplexes. Protein samples were harvested from 24-96 hours post-transfection and then run on 10% polyacrylamide gels before Western blot analysis for Oct4 expression levels. GAPDH was used as a loading control.

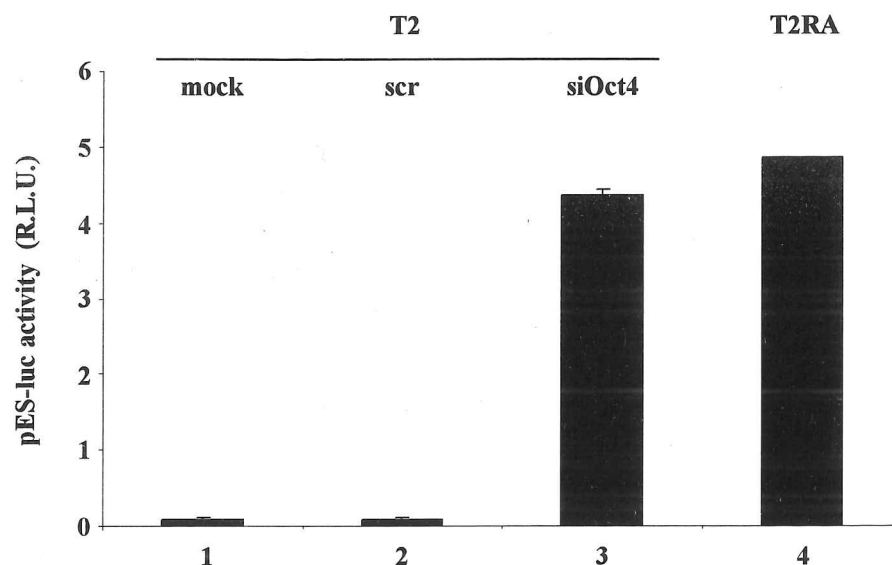


Figure 5.12 Down-regulation of Oct4 in T2 cells is associated with an increase in MIEP activity. T2 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or Oct4-specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected T2 cells and differentiated T2RA cells (lane 4) were transfected with pES-luc. After 24 hours, cells were harvested and MIEP activity analysed by luciferase assay. Analysis was normalised by co-transfection with β -gal. (R.L.U. = relative light units). *Error bars*, 1 S.D. of three independent experiments.

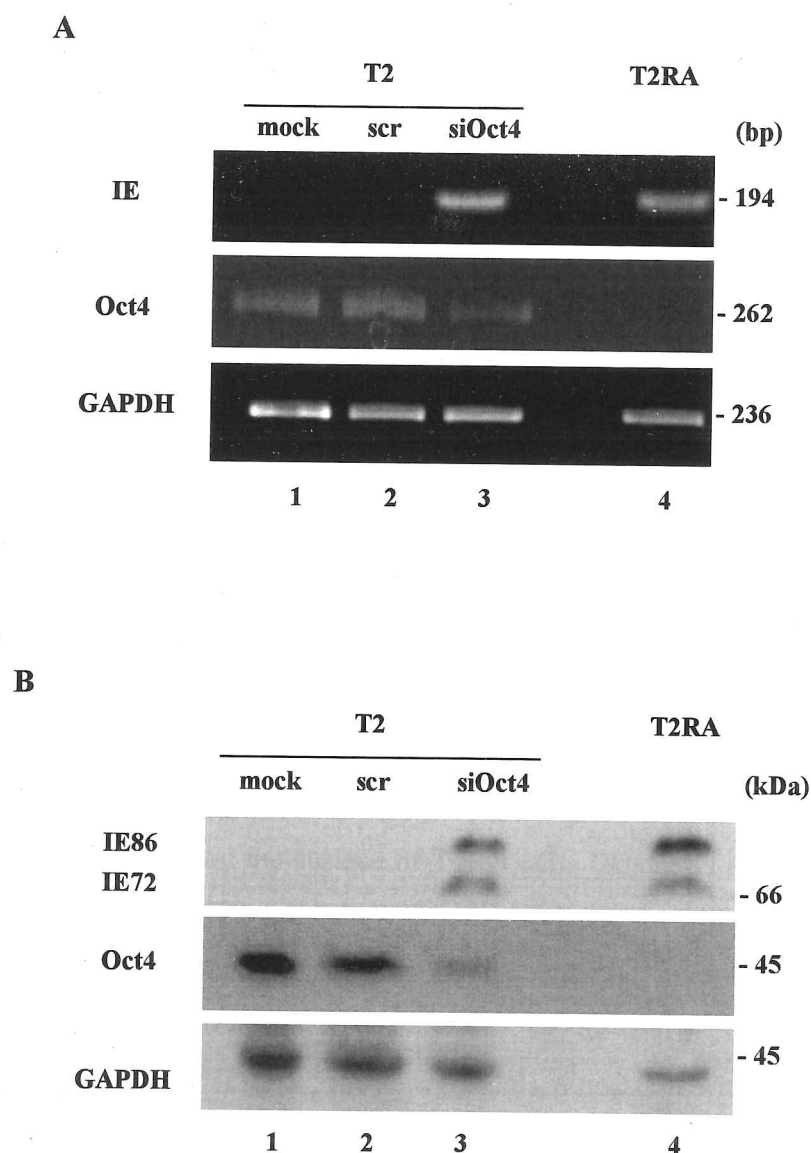


Figure 5.13 Down-regulation of Oct4 in T2 cells leads to an induction of viral IE gene expression upon HCMV infection. T2 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or hDaxx-specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected T2 cells and differentiated T2RA cells (lane 4) were infected with HCMV (MOI 0.5) for 24 hours. (A) Cells were then harvested and total RNA extracted. After reverse transcription, specific PCR was carried out for viral IE genes and Oct4. GAPDH was used as a loading control. (B) Cells were harvested and protein content analysed by Western blot for viral IE and Oct4 expression. GAPDH was used as a loading control.

levels of IE72/86 expression were observed with differentiated T2RA cells, as expected (Fig. 5.14c, upper panel).

Interestingly, the nuclear morphology of siOct4-transfected T2 cells (Fig. 5.14b, lower panel) was quite similar to differentiated T2RA cells (Fig. 5.14c, lower panel), which was not the case for scramble siRNA transfected T2 cells (Fig. 5.14a, lower panel). This would be consistent with recent reports which suggest that siRNA down-regulation of Oct4 in ES cells and T2 cells can result in full cellular differentiation (Hay *et al.*, 2004; Matin *et al.*, 2004). It is possible that Oct4 down-regulation by siRNA might induce viral IE gene expression not because it results in terminal differentiation of the T2 cells but because Oct4 is an intrinsic repressor of the viral MIEP. However, computational analysis of the MIEP using TFsearch for Oct4 binding sites (ATGCAAAT) did not identify any within the promoter. To confirm that down-regulation of Oct4 did result in differentiation of T2 cells, cells transfected with scramble siRNA or siOct4-specific siRNAs for 72 hours and T2RA cells (as a positive control for differentiation) were fixed and stained for the surface expression of SSEA3 (red), a globoseries glycolipid antigen present on undifferentiated T2 cells that is down-regulated on the surface of T2RA cells (Andrews *et al.*, 1984). Whereas scramble siRNA transfected T2 cells show positive staining for SSEA3 (Fig 5.15a, upper panel), T2RA cells were negative for this undifferentiated marker (Fig. 5.15c, upper panel), as expected (Murphy *et al.*, 2002). However, siOct4-transfected T2 cells were also negative for SSEA3 staining (Fig. 5.15b, upper panel), consistent with down-regulation of Oct4 in these cells resulting in full differentiation, consistent with results of other groups (Hay *et al.*, 2004; Matin *et al.*, 2004).

5.2.7 Re-expression of Oct4 in permissive T2RA cells is not associated with repression of IE gene expression

As well as the down-regulation of Oct4 in T2 cells upon differentiation of T2RA cells, it was also known that the histone deacetylases HDAC3 also decreases in expression after 6 days with RA treatment (Murphy *et al.*, 2002). These studies also showed that re-expression of HDAC3 in differentiated T2RA cells decreased the MIEP activity after transfection or by analysis of infection efficiency (Murphy *et al.*, 2002). Therefore, it was next investigated whether Oct4 re-expression in normally permissive T2RA cells could cause a decrease or loss of infectivity as seen with HDAC3 re-expression. T2RA cells were

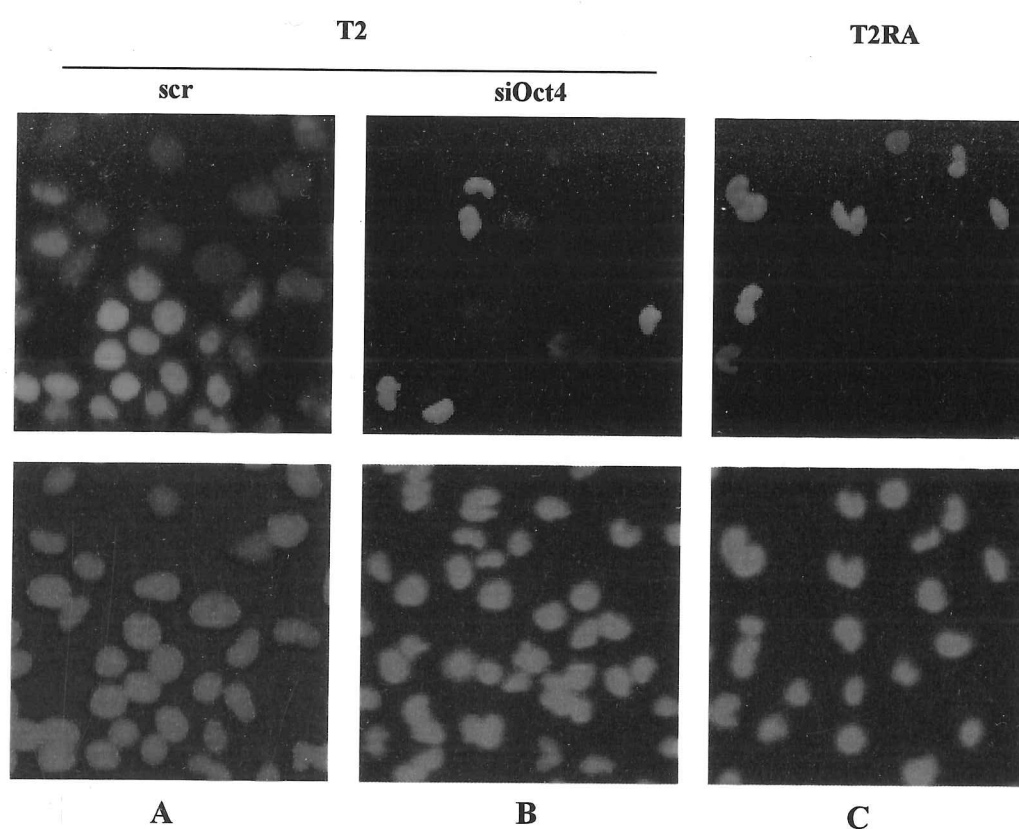


Figure 5.14 Down-regulation of Oct4 in T2 cells is associated with permissiveness to HCMV infection. T2 cells were transfected with either (A) scramble or (B) Oct4-specific oligonucleotide duplexes. After 72 hours, scramble and siOct4-transfected T2 cells and (C) T2RA cells were infected with HCMV Toledo (MOI 0.5). After 24 hours, cells were fixed, permeabilised and stained for IE72/86 (red) and Oct4 (green) expression. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.

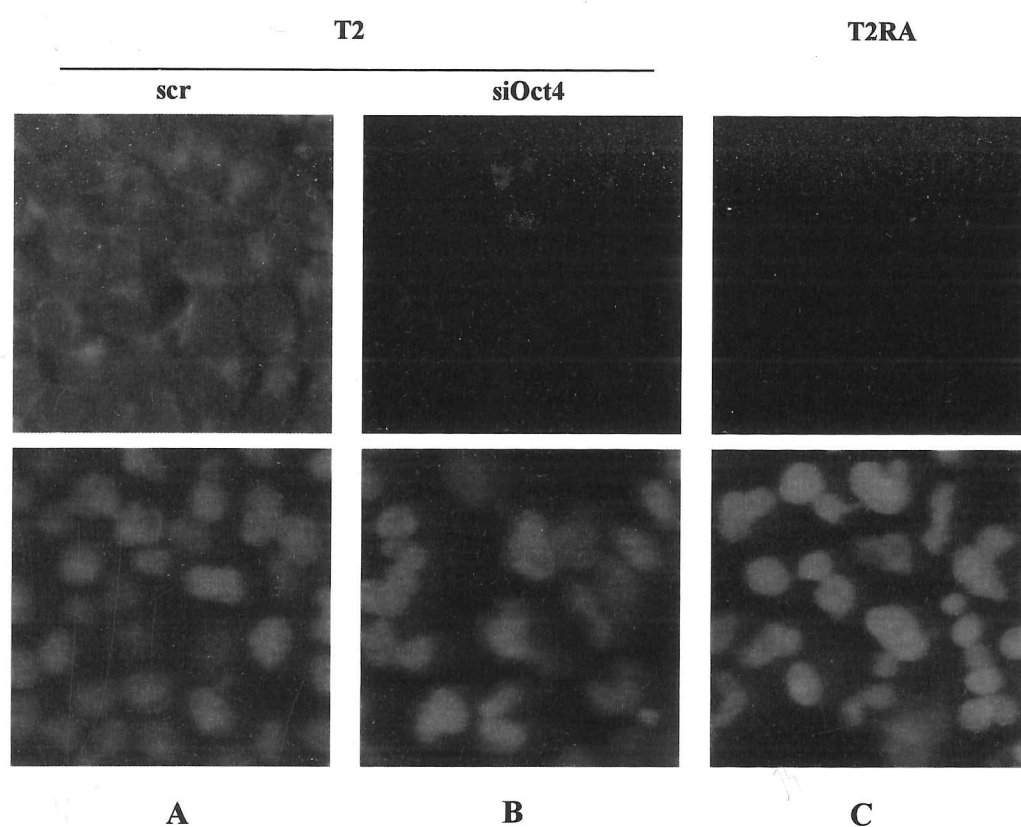


Figure 5.15 Down-regulation of Oct4 in T2 cells is associated with a loss of the undifferentiated marker SSEA3. T2 cells were transfected with either (A) scramble or (B) Oct4-specific oligonucleotide duplexes. After 72 hours, scramble and siOct4-transfected T2 cells and (C) T2RA cells were fixed and stained for SSEA3 surface expression (red). Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.

mock transfected (Fig. 5.16, lane 2), transfected with pcDNA3 control (Fig. 5.16, lane 3) or pCMV-Oct4 (pOct4) (Fig. 5.16, lane 4). After 24 hours, transfected T2RA cells and T2 cells (Fig. 5.16, lane 1) were infected with HCMV Toledo (MOI 0.5). After a further 24 hours, total RNA and protein samples were harvested from each population. RT-PCR was used to quantify IE and Oct4 RNA. GAPDH was used as a loading control. Concurrently, Western blot analysis was employed to determine the levels of protein. Transfection of T2RA cells with pOct4 resulted in levels of re-expression of Oct4 to those observed in T2 cells (Fig. 5.16, lane 4 & 1 respectively) compared to control pcDNA3 transfected cells (Fig. 5.16, lane 3). However, it was noticeable that re-expression of Oct4 in T2RA cells resulted in a lower molecular weight form of Oct4 on Western blot analysis (Fig. 5.16b, lane 4). Nevertheless, this re-expression of Oct4 did not result in any effect on the permissiveness of cells for IE gene expression at the level of RNA (Fig. 5.16a, lane 4) or protein (Fig. 5.16b, lane 4).

Even though re-expression of Oct4 had no effect on IE gene expression after virus infection, it was next assessed whether this lack of an effect could be recapitulated after transfection of the MIEP. T2RA cells were treated as above before they were transfected, as well as T2 cells, with pES-luc. After 24 hours, cells were then harvested and MIEP activity analysed by luciferase assay, with normalisation of data by β -gal co-transfection (Fig. 5.17). Although slight variation in MIEP activity was seen above the level of mock transfection (Fig. 5.17, lane 2) with pcDNA3 (~11%) (Fig. 5.17, lane 3) or pOct4 transfection (~8%) (Fig. 5.17, lane 4), in comparison to control T2 cells (Fig. 5.17, lane 1) no significant effect upon MIEP activity was observed. Finally, indirect immunofluorescence was employed to determine any effect of Oct4 re-expression on infection at the cell level (Fig. 5.18). T2RA cells were again transfected as above before infection with HCMV Toledo (MOI 0.5). After 24 hours, cells were fixed, permeabilised and stained for Oct4 (green) and viral IE protein (red). Control transfected T2RA cells (Fig. 5.18a & b, upper panels) showed no Oct4 expression, as expected, after differentiation. In comparison, pOct4 transfected T2RA cells (Fig. 5.18c, upper panel) showed clear Oct4 expression in a number of cells and this co-localised with IE72/86. Therefore, Oct4 re-expression in T2RA cells did not result in any detectable decrease in permissiveness for HCMV infection.

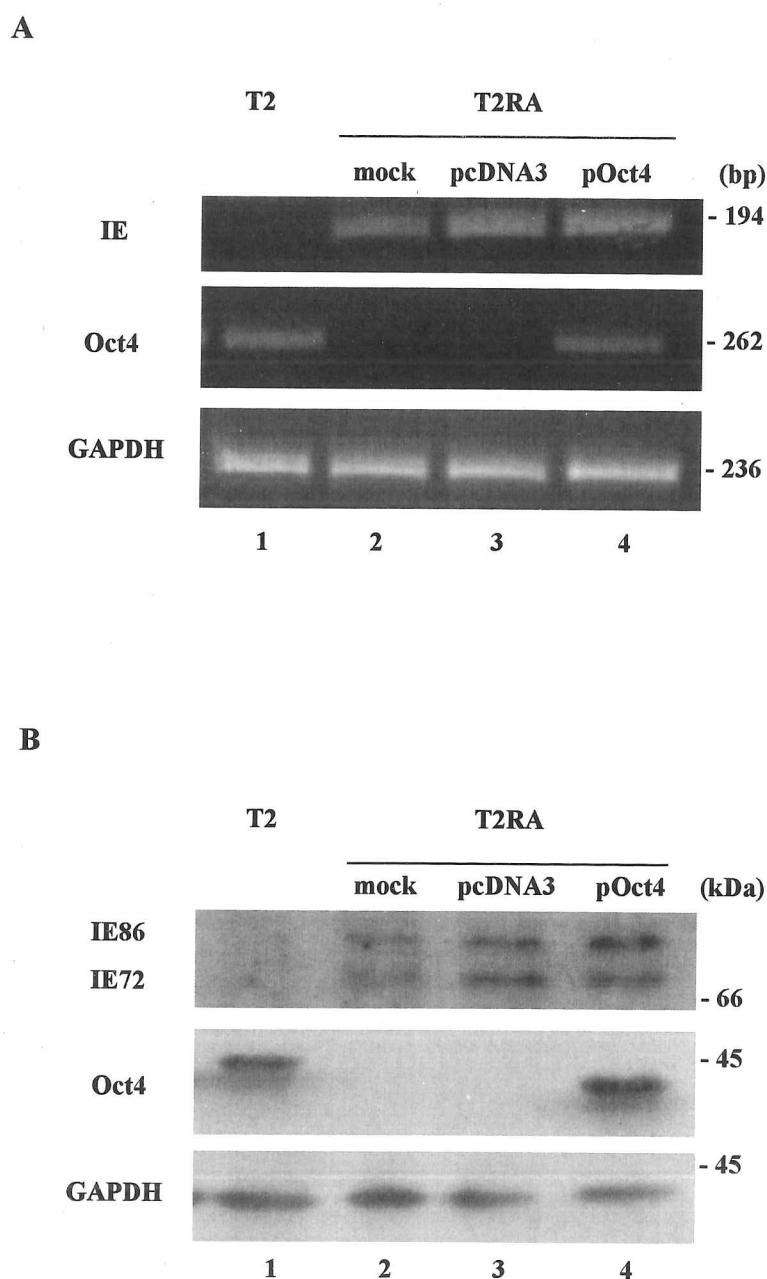


Figure 5.16 Re-expression of Oct4 in T2RA cells is not associated with a repression of viral IE gene expression. T2RA cells were either mock transfected (lane 2) or transfected with pcDNA3 (lane 3) or pCMV-Oct4 (pOct4; lane 4). After 24 hours, transfected T2RA cells and undifferentiated T2 cells (lane 1) were infected with HCMV (MOI 0.5) for 24 hours. (A) Cells were then harvested and total RNA extracted. After reverse transcription, specific PCR was carried out for viral IE genes and Oct4. GAPDH was used as a loading control. (B) Cells were harvested and protein content analysed by Western blot for viral IE and Oct4 expression. GAPDH was used as a loading control.

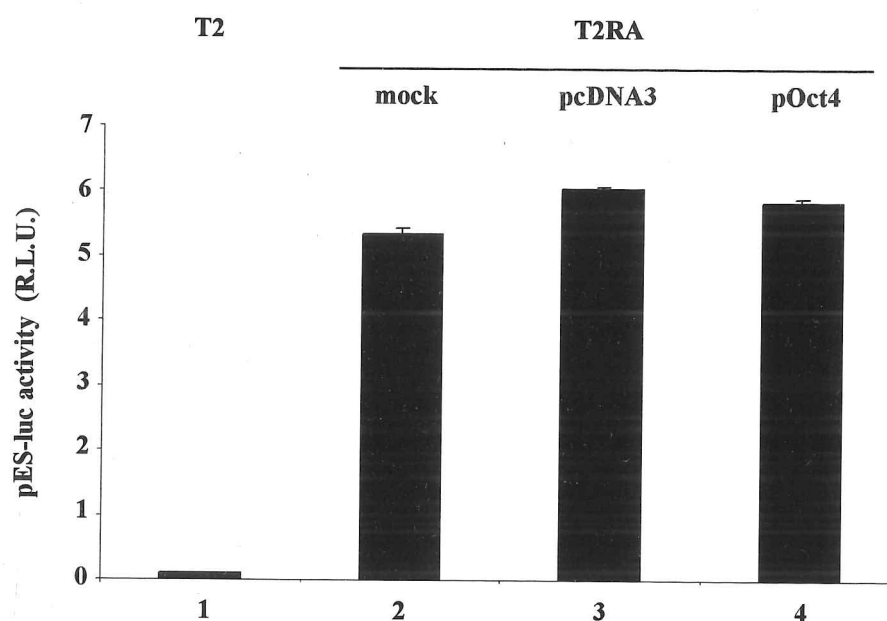


Figure 5.17 Re-expression of Oct4 in T2RA cells is not associated with a repression of MIEP activity. T2RA cells were either mock transfected (lane 2) or transfected with pcDNA3 (lane 3) or pCMV-Oct4 (pOct4; lane 4). After 24 hours, transfected T2RA cells and undifferentiated T2 cells (lane 1) were transfected with pES-luc. After a further 24 hours, cells were harvested and MIEP activity analysed by luciferase assay. Analysis was normalised by co-transfection with β -gal. (R.L.U. = relative light units). Error bars, 1 S.D. of three independent experiments.

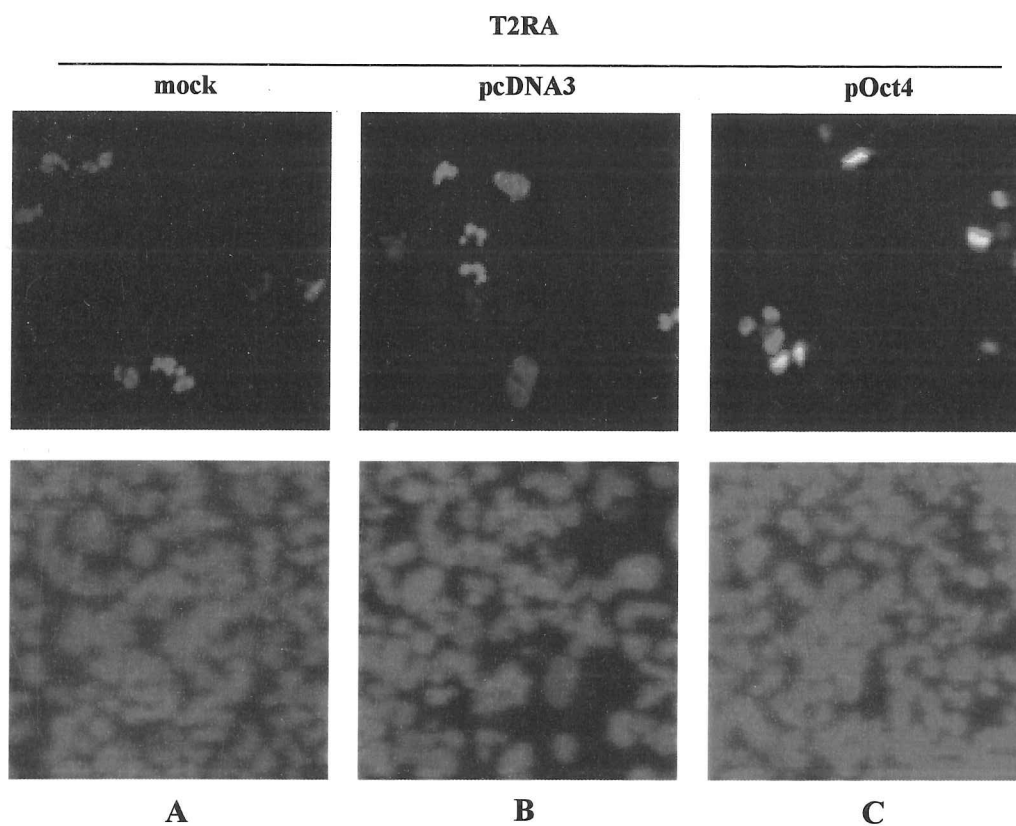


Figure 5.18 Re-expression of Oct4 in T2RA cells is not associated with abrogation of HCMV IE gene infection. T2RA cells were either (A) mock transfected or transfected with either (B) pcDNA3 or (C) pCMV-Oct4. After 24 hours, T2RA cells were infected with HCMV Toledo (MOI 0.5). After a further 24 hours, cells were fixed, permeabilised and stained for IE72/86 (red) and Oct4 (green) expression. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.

5.2.8 Down-regulation of HP1 β in T2 cells is not associated with an induction of IE gene expression

Inhibition of hDaxx expression by siRNA did not affect HCMV IE gene expression or the chromatin structure of the MIEP after infection: the MIEP was predominantly associated with HP1 β , a marker of transcriptional repression (Bannister *et al.*, 2001; Kouzarides, 2007a). However, previous work has shown pan-specific inhibition of HDACs by TSA results in permissiveness of undifferentiated T2 cells for IE gene expression and virus production (Meier, 2001; Murphy *et al.*, 2002). Other reports have also suggested that HDAC inhibitors can be used to reactivate other viruses from quiescence: the LTRs of the integrated provirus of HIV are known to be chromatinised and repressed via HDAC recruitment (Coull *et al.*, 2000; Williams *et al.*, 2006) and TSA treatment activates LTR-driven transcription (Van Lint *et al.*, 1996). Interestingly, it appears that all isoforms of HP1 are associated with the repression of the HIV proviral LTRs (Marban *et al.*, 2007) and that a siRNA, directed specifically to HP1 γ , results in virus reactivation (du Chene *et al.*, 2007). As down-regulation of HP1 β can result in an increase in MIEP-driven transcription after transfection (du Chene *et al.*, 2007), the possibility that down-regulation of HP1 β in T2 cells might induce IE gene expression was investigated.

T2 cells were mock transfected (Fig. 5.19, lane 1) or transfected with scramble siRNA (Fig. 5.19, lane 2) or HP1 β -specific (Fig. 5.19, lane 3) siRNA. After 72 hours, transfected T2 cells as well as differentiated T2RA cells (Fig. 5.19, lane 4) were infected with HCMV Toledo (MOI 0.5) for 24 hours. Total cell RNA and protein was analysed for levels of expression of HP1 β and Oct4 by RT-PCR or Western blot analysis, respectively. The HP1 β RNA and protein level was shown to decrease substantially in siHP1 β -transfected cells (Fig. 5.19, lane 3) compared to the mock and scramble siRNA controls (Fig. 5.19, lanes 1 & 2) consistent with previous work (du Chene *et al.*, 2007). Additionally, this down-regulation of HP1 β had no effect on the differentiation state of T2 cells on the basis of expression of Oct4 (Fig. 5.19, lane 3). However, no induction of IE RNA (Fig. 5.19a, lane 3) or protein (Fig. 5.19b, lane 3) occurred in HP1 β down-regulated T2 cells by 24 hours post-infection. In contrast, T2RA cells showed both an absence of Oct4 expression due to differentiation and the presence of both IE RNA (Fig. 5.19a, lane 4) and protein (Fig. 5.19b, lane 4) upon infection, as expected.

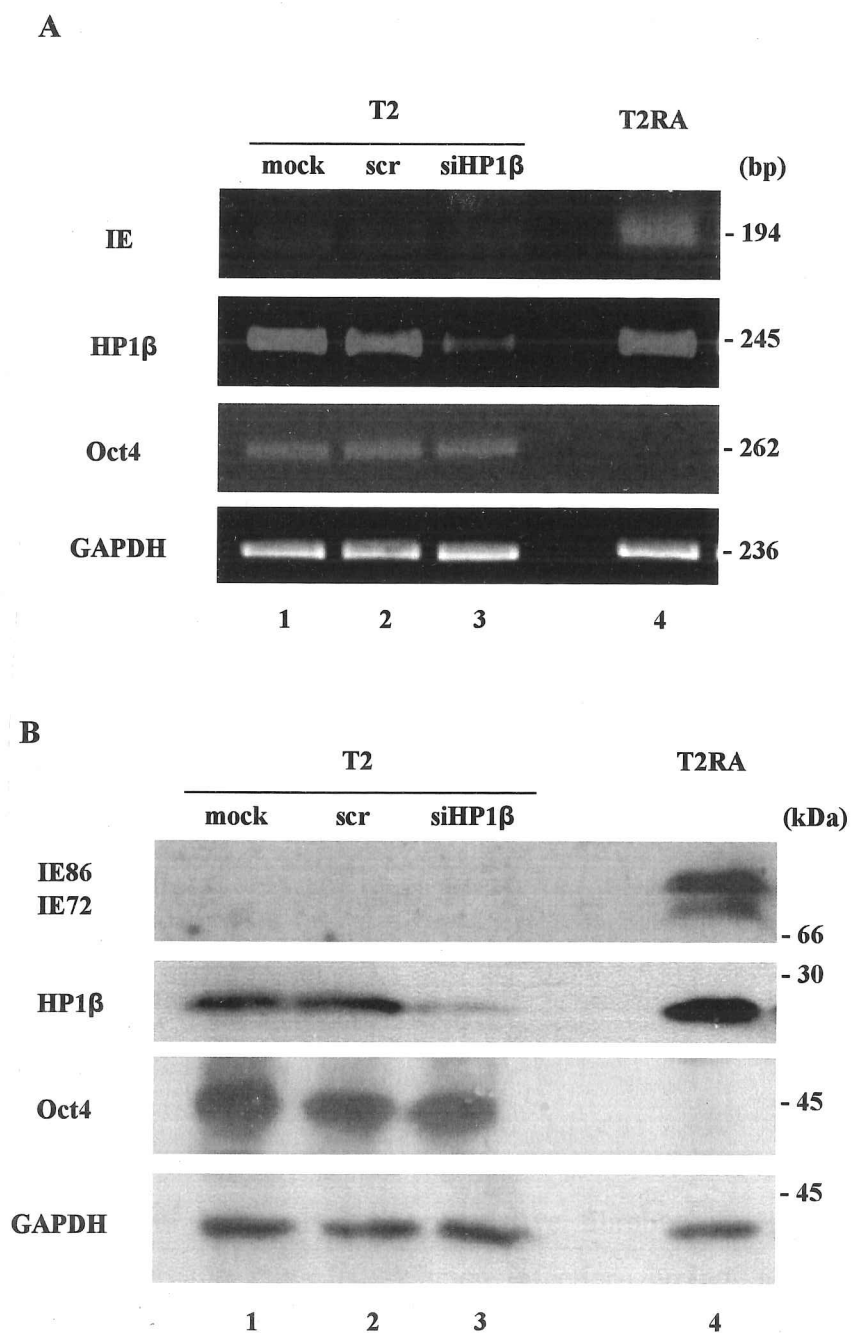


Figure 5.19 Transient down-regulation of HP1 β in T2 cells has no effect on viral IE gene expression upon HCMV infection. T2 cells were either mock transfected (lane 1) or transfected with scramble (lane 2) or HP1 β -specific (lane 3) oligonucleotide duplexes. After 72 hours, transfected T2 cells and differentiated T2RA cells (lane 4) were infected with HCMV Toledo (MOI 0.5) for 24 hours. (A) Cells were then harvested and total RNA extracted. After reverse transcription, specific PCR was carried out for viral IE genes, Oct4 and HP1 β . GAPDH was used as a loading control. (B) Cells were harvested and protein content analysed by Western blot for viral IE, Oct4 and HP1 β expression. GAPDH was used as a loading control.

Indirect immunofluorescence was next used to determine if the RT-PCR and Western blot results of HP1 β down-regulation could be reflected at the cell level (Fig. 5.20 & 5.21). As scramble siRNA transfected T2 cells again appeared to behave as mock transfected cells, these were solely employed as a control. T2 cells were either transfected with scramble siRNA (Fig. 5.20a) or HP1 β -specific (Fig. 5.20b) siRNAs. After 72 hours, cell populations were fixed, permeabilised and stained for HP1 β (red): Hoechst 33342 was used to counter-stain nuclei. Consistent with the RNA and protein data, the level of down-regulation of HP1 β in siHP1 β -transfected T2 cells was substantial (Fig. 5.20b, upper panel) when compared to scramble siRNA transfected cells (Fig. 5.20b, upper panel). In addition, differentiated T2RA cells showed a similar level of expression of HP1 β (Fig. 5.20c, upper panel). Therefore, examination of the effects of HP1 β down-regulation on IE gene expression was next investigated (Fig. 5.21). T2 cells were treated with scramble siRNA or HP1 β -specific siRNA as above before infection with HCMV Toledo (MOI 0.5). After 24 hours, cells were fixed, permeabilised and stained for IE72/86 (red), and Oct4 (green) protein: Hoechst 33342 was again used to counter-stain nuclei. HP1 β down-regulation resulted in no changes in the expression of Oct4 in T2 cells and was not associated with any induction of IE gene expression (Fig. 5.21b, upper panel), when compared to scramble siRNA transfected control (Fig. 5.21a, upper panel). In contrast, T2RA cells appeared fully permissive for IE gene expression (Fig. 5.21c, upper panel), as expected. Thus, HP1 β down-regulation appears to have little effect on the permissiveness of T2 cells to HCMV infection.

5.3 Discussion

The down-regulation of hDaxx in fully permissive fibroblasts relieved repression of HCMV IE gene expression immediately upon infection through a loss of repressive chromatin at the MIEP (see Chapter 4; Woodhall *et al.*, 2006). In addition, expression of viral IE genes after infection of conditionally permissive cell types with HCMV is known to be regulated by chromatin structure at the MIEP (Murphy *et al.*, 2002; Reeves *et al.*, 2005a; Reeves *et al.*, 2005b; Ioudinkova *et al.*, 2006). Therefore, whether hDaxx had any role in establishing a non-permissive environment for HCMV infection in undifferentiated cells and thus could play any part in mediation of HCMV latency or reactivation was a key question.

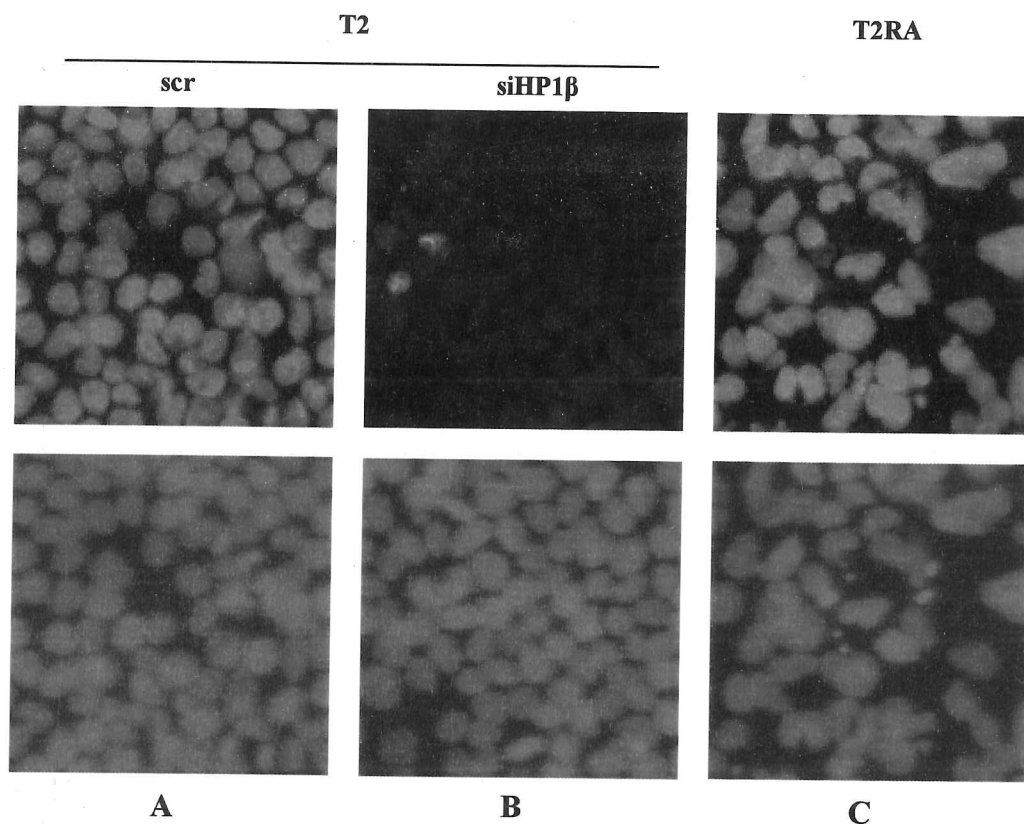


Figure 5.20 HP1 β -specific siRNA oligonucleotides cause down-regulation of HP1 β in T2 cells. T2 cells were transfected with either (A) scramble or (B) HP1 β -specific oligonucleotide duplexes. After 72 hours, transfected T2 cells and T2RA cells (C) were fixed, permeabilised and stained for HP1 β (red) expression. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.

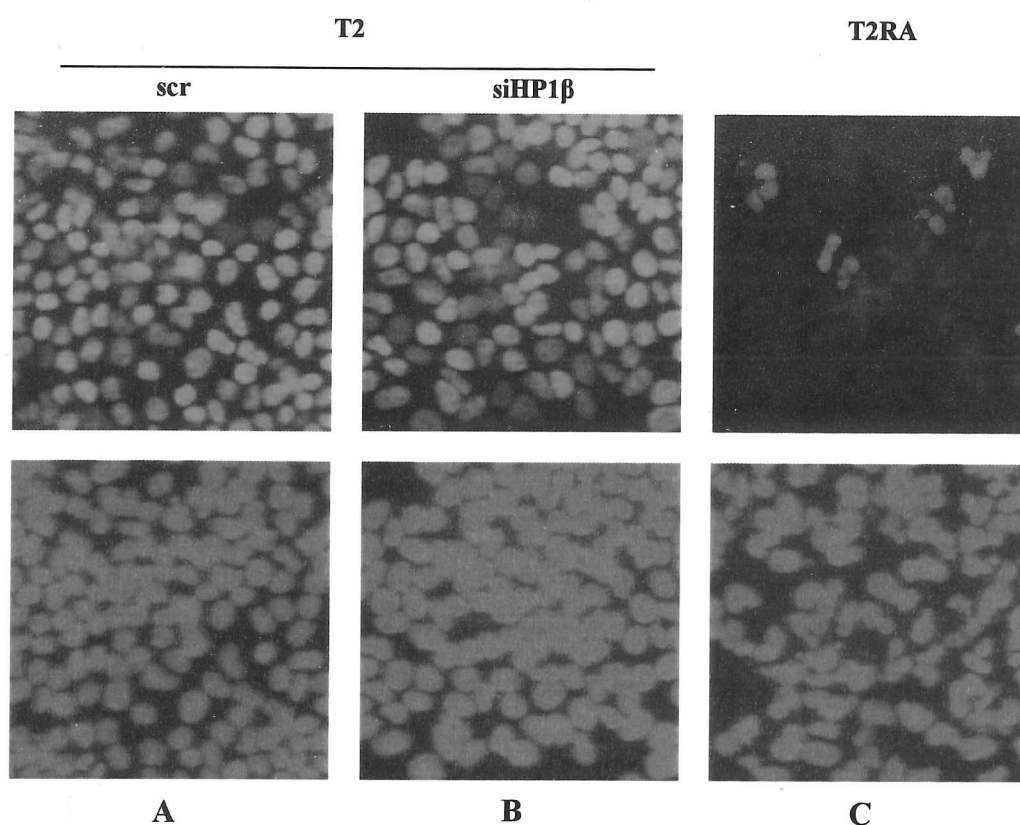


Figure 5.21 Transient down-regulation of HP1 β in T2 cells has no effect on viral IE gene expression upon HCMV infection. T2 cells were transfected with either (A) scramble or (B) HP1 β -specific oligonucleotide duplexes. After 72 hours, scramble and siHP1 β transfected T2 cells and (C) T2RA cells were infected with HCMV Toledo (MOI 0.5). After 24 hours, cells were fixed, permeabilised and stained for viral IE (red) and Oct4 (green) expression. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.

Clearly, differentiation of both monocytes *ex vivo* and conditionally permissive cell models leads to permissiveness for HCMV IE gene expression (Murphy *et al.*, 2002; Reeves *et al.*, 2005a; Reeves *et al.*, 2005b). Therefore, if hDaxx is involved in mediating repression in undifferentiated cells, it is possible that the steady state levels of hDaxx protein would decrease during and after differentiation. However, hDaxx protein levels did not change significantly throughout differentiation of monocytes to mature DCs or during differentiation of T2 cells to T2RA cells. Similarly, levels of hDaxx did not change during the differentiation of THP1 cells to macrophages consistent with levels of hDaxx expression having little correlation to differentiation status and hence regulation of IE gene expression. hDaxx down-regulation caused no change to the differentiation state of the T2 cells, as assessed by Oct4 expression, nor did it lead to any induction of IE gene expression. Although residual amounts of hDaxx protein were still present in hDaxx knockdown T2 cells, this was unlikely to be responsible for maintaining the repression of IE gene expression as this level of residual hDaxx was not sufficient to repress a known hDaxx-repressed promoter.

Despite the convincing result that hDaxx down-regulation had no effect upon HCMV IE gene expression within undifferentiated T2 cells, it remained that this could be due to a peculiarity of the strain of HCMV or MOI used to infect cells. However, further analogous experiments carried out using the highly passaged laboratory strain HCMV AD169 at a high MOI failed to result in any induction of IE gene expression. Indeed, the activity of the MIEP in isolation, as determined by luciferase assay, showed no change with hDaxx down-regulation in undifferentiated T2 cells. Although the use of a likely activator, such as the HDAC inhibitor TSA, was not used here to confirm the viability of the system (as had been before: Murphy *et al.*, 2002), use of down-regulation of Oct4 discussed later did confirm that our siRNA technology could result in T2 cells becoming permissive for IE gene expression.

T2 cells are known to spontaneously differentiate to T2RA like cells if their culture environment is not optimal. Therefore, to assess cells individually, indirect immunofluorescence was employed. The presence or down-regulation of hDaxx cannot be verified by indirect immunofluorescence in T2 cells, as in fibroblasts (see Chapter 4), due to the low level of nucleated protein at abnormal ND10 (Hsu & Everett, 2001). However, it

is clear that hDaxx down-regulation firstly does not lead to any differentiation and secondly does not cause an induction of IE gene expression. Consistent with this, subsequent analysis of the chromatin structure of the viral MIEP found little to no difference in the chromatin state with hDaxx knockdown T2 cells. Hence, down-regulation of hDaxx in T2 cells had been shown unequivocally to cause no change to the MIEP in those infected cells concomitant with a lack of induction of IE gene expression.

The observation that hDaxx down-regulation in T2 cells does not lead to an induction of IE gene expression (Groves & Sinclair, 2007) is inconsistent with a recently published parallel study by Saffert & Kalejta (2007). In this analysis, hDaxx down-regulation in NTera-2 cells, the parental line from which the NT2D1 (T2) cell line was cloned, resulted in permissive infection of these cells with HCMV. The reasons for this discrepancy are unclear. However, Saffert & Kalejta's work employed the highly passaged laboratory isolate of HCMV, AD169, which has known genome deletions and has lost tropism for other cells such as monocytes and endothelial cells. In addition, the NTera-2 cell line is known to regularly spontaneously differentiate (Kalejta, personal communication) and it is interesting to note that in their hDaxx knockdown cells there did appear to be a reduction in Oct4 expression, suggesting their cells may have become partially differentiated, and a low background level of IE gene expression occurring in apparently undifferentiated cell populations (Saffert & Kalejta, 2006).

Interestingly, although the work of Saffert & Kalejta demonstrates an induction of HCMV IE gene expression in hDaxx down-regulated NTera-2 cells and THP1 cells, again in contrast to the data presented in this chapter, viral early gene expression (UL44) was reduced in these cells and there was also a block in late gene expression and the production of infectious virus. This is consistent with another study where ectopic expression of IE72 and IE86 can cause induction of early gene expression but not full virus replication in THP1 cells (Yee *et al.*, 2007). It is questionable though whether hDaxx plays any role in the establishment of latency or maintenance of repression in NTera-2 or T2 cells since they do not appear to have normal ND10 (Hsu & Everett, 2001). Therefore, it is likely that the differentiation-dependent transcriptional milieu is the over-riding factor which determines whether a cell is permissive or not for HCMV IE gene expression and productive infection.

The differentiation-dependent regulation of IE gene expression in T2 cells paralleled Oct4 expression levels. Oct4, which is also known as POU5F1, Oct3 or Oct3/4, is a master regulator of differentiation and used routinely as a marker for undifferentiated ES cells (Rosner *et al.*, 2000; Deb-Rinker *et al.*, 2005). All members of the Oct family contain two highly conserved domains, a POU homeodomain and a POU-specific domain, that are separated by 14 to 26 variable amino acids. These domains are required for DNA binding and are involved in protein-protein interactions. Intriguingly, a number of other octamer proteins have been implicated in the regulation of herpesvirus IE gene expression. Oct1 (POU2F1) has been highlighted as playing an important and definite role in HSV-1 transcriptional activation (Efsthathiou & Preston, 2005) (see Introduction). During HCMV infection of GM-Ps, Oct2 (POU2F2) is up-regulated and the authors suggest that this may aid repression of IE gene expression, thus favouring establishment of latency (Slobedman *et al.*, 2004). Similarly, Oct2 has also been suggested to be involved in repression of HSV-1 and VZV IE gene expression in other models (Lillicrop *et al.*, 1991; Patel *et al.*, 1998). In T2 cells, Oct1 expression can cause an increase in MIEP activity when analysed using transfection assays and Oct6 expression can cause MIEP repression (Mendelson thesis, 1997).

Although a computational analysis of the MIEP using TFSearch did not identify Oct4 binding sites (ATGCAAAT), differentiation of T2 cells and their concomitant permissiveness for HCMV IE gene expression coincided with a decrease in Oct4 expression. Therefore, it was hypothesised that Oct4 could be acting as a repressive transcription factor in undifferentiated T2 cells. Down-regulation of Oct4 by siRNA treatment in T2 cells led to an induction of MIEP activity and of expression of IE RNA and protein. Therefore, it appeared that Oct4 may have some role in directly controlling the activity of the MIEP during HCMV infection in T2 cells. However, it was found that the morphology of the nuclei of Oct4 knockdown T2 cells changed to that more reminiscent of differentiated T2RA cells. Hence, it was possible that Oct4 down-regulation in T2 cells was actually leading to differentiation of this cell type and subsequently causing permissiveness to HCMV infection. Also, recently published work had shown that Oct4 down-regulation in T2 and ES cells lines led to differentiation (Hay *et al.*, 2004; Matin *et al.*, 2004). Indeed, the absence of surface expression of SSEA3 concluded that down-

regulation of Oct4 in T2 cells had caused differentiation, consistent with permissiveness of these cells for HCMV infection.

Recently, the validity of using Oct4 as a marker for undifferentiated ES cells has been questioned as some adult mammalian differentiated cells continue to express Oct4 (Zangrossi *et al.*, 2007). Additionally, Oct4 expression appears not to be necessary for mouse somatic stem cell self-renewal (Lengner *et al.*, 2007). However, the use of Oct4 as a marker of differentiation of the T2 cell line is routinely accepted. Indeed, it is interesting to note that Oct4 expression has also been found in the PBMC of humans (Tai *et al.*, 2005). Although, it remains to be determined whether similar Oct4 down-regulation of an as yet unspecified PBMC sub-population may lead to permissive infection with HCMV.

Work from our laboratory has previously demonstrated that the re-expression of HDAC3, which decreases upon differentiation of T2 cells, in T2RA cells is sufficient to cause repression of IE gene expression (Murphy *et al.*, 2002). However, analogous experiments re-expressing Oct4 in T2RA cells found no inhibition of MIEP activity or IE gene expression during HCMV infection. This data is consistent with the above finding that it is the differentiation status and the associated levels of factors in the infected cell which are important for the permissiveness of HCMV infection. It is possible that the re-introduction of other additional factors to T2RA cells would be necessary to cause repression of MIEP activity. Recently published data from several laboratories has shown that the re-expression of certain factors including Oct4 can lead to the production of pluripotent stem cells from once differentiated cells. The expression of Oct4, Sox2, c-Myc and Klf4 in fibroblast cultures was first shown to direct the generation of pluripotent murine stem cells (Takahashi & Yamanaka, 2006; Wernig *et al.*, 2007) whilst the further inclusion of Nanog resulted in the selection of germ-line competent induced pluripotent stem (iPS) cells (Okita *et al.*, 2007). This work was followed by production of iPS cells from adult human somatic dermal fibroblasts through re-expression of either Oct4, Sox2, Klf4 and c-Myc (Takahashi *et al.*, 2007) or Oct4, Sox2, Nanog and LIN28 (Yu *et al.*, 2007a). Most recently, re-expression of Oct4, Sox2, Klf4 and c-Myc in fetal, neo-natal and adult human primary cells (including dermal fibroblasts) has provided iPS cells, with Lin28 and Nanog complementing this reprogramming event (Park *et al.*, 2008). Overall, this has led to an exciting new method of developing pluripotent stem cells specific for an individual which,

hypothetically, could be used at a therapeutic level against many human diseases. Although Oct4 re-expression alone did not lead to a repression of HCMV infection due to de-differentiation of the cell type, it is possible that a similar subset of factors could be used to reprogram these cells to cause this effect. However, this so far remains untested and it is unknown whether this may be possible within a neuroblastoma cell line.

As down-regulation of hDaxx had proved insufficient for induction of IE gene expression, other factors were investigated for a possible role in the establishment of latency or regulation of permissiveness of HCMV infection within the T2 cell line. Recently, it has been established that the induction of gene expression from the LTR of certain retroviruses can be repressed by HDAC recruitment and is associated with all HP1 isoforms; relief of this repression has been shown through the use of RNAi technology or TSA treatment (Coull *et al.*, 2000; Williams *et al.*, 2006; du Chene *et al.*, 2007; Marban *et al.*, 2007; Poleshko *et al.*, 2008). In addition, HP1 β down-regulation has also specifically caused an increase in the activity of the MIEP in isolation (du Chene *et al.*, 2007). However, the down-regulation of HP1 β in T2 cells caused no induction of IE gene expression during HCMV infection. Therefore, it remains that further repressive features of the chromatin structure (di- and tri-methylated lysine 9 on histone-H3) and the presence of factors, such as HDACs, able to repress the MIEP may still be sufficient to inhibit the induction of IE gene expression even with HP1 β down-regulation in T2 cells.

Overall, the work in this chapter demonstrates that down-regulation of factors such as hDaxx and HP1 β is not sufficient for induction of IE gene expression to take place in HCMV infected, undifferentiated cell types. Indeed, the down-regulation of the master regulator of differentiation Oct4 further substantiates the understanding that HCMV IE gene expression is ultimately dependent upon the differentiation status of the infected cell and that it is changes to the transcriptional milieu that is ultimately responsible for the permissiveness of infection within these cells.

6. Results

All classes of HCMV gene promoters are subject to chromatinisation and remodelling throughout productive infection

6.1 Introduction

Work presented in chapters 3, 4 and 5, as well as other published data, has established a clear role for the modulation of chromatin structure at the viral MIEP during reactivation from latency and full productive infection. This form of control of transcription has also been shown to be important in the regulation of a number of other DNA viruses (described in section 1.11) including the prototypic alphaherpesvirus HSV-1 (reviewed in: Knipe & Cliffe, 2008). Studies have shown that, as with HCMV, the HSV-1 genome is associated with histones and repressive chromatin during latent infection of various tissues and model systems which is relieved upon reactivation of the virus (Dressler *et al.*, 1987; Deshmane & Fraser, 1989; Arthur *et al.*, 2001; Bloom, 2004; Kubat *et al.*, 2004; Wang *et al.*, 2005; Amelio *et al.*, 2006). More recently, it has been shown that the promoters of certain HSV-1 genes are also associated with histones throughout productive, lytic infection and are modulated throughout infection in a temporally controlled manner (Herrera & Triezenberg, 2004; Kent *et al.*, 2004).

Investigations into chromatin remodelling at HSV-1 promoters throughout lytic infection have established that the promoters are remodelled in a temporally regulated fashion consistent with expression of those genes examined. This has been demonstrated by two studies where investigation of the genes and promoters of archetypal examples of each class of herpesvirus gene have been employed: during lytic infection, the genes and promoters of ICP0 (IE), thymidine kinase (early) and VP16 (early/late) were found to be associated with acetylation of lysine 9 on histone-H3 in a temporally associated fashion and, in addition, H3K9me2 was associated with the ICP0 promoter at late times of infection (Kent *et al.*, 2004). Furthermore, analysis of HSV-1 promoters during reactivation from quiescence via exogenous ICP0 expression through HSV-2 super-infection demonstrated the additional association of HP1 α after increases at H3K9me3 at temporally controlled times of infection on the promoters of ICP0 (IE), ICP27 (IE) and gC (late) whilst the chromatin status of the Lat region was reversed (Coleman *et al.*, 2008).

During investigation of chromatin association with the MIEP during HCMV productive infection in this study, it was demonstrated that negative regulation of viral IE gene expression was prevented by the use of HDAC inhibitors (Chapter 3) and also by hDaxx down-regulation (Chapter 4). As a consequence of this loss of repression, an increase in the level of IE gene expression occurred. However, it was also apparent that the subsequent expression of HCMV early and late genes was also increased in a similar fashion. During the beginning of this investigation into the involvement of chromatin remodelling and the control of HCMV gene expression, work from another group was published also consistent with the aforementioned observations. During a study of the involvement of major IE proteins and HDAC in the regulation of HCMV gene expression, ChIP analysis of both the MIEP and the UL44 promoter demonstrated an increase in the level of acetylated histone-H4 association from 1 to 12 hours post-infection (Nevels *et al.*, 2004b). This suggested that promoters other than the MIEP were also associated with histone proteins and that the regulation of their transcription appeared to involve histone modification during the first 12 hours of infection. Consequently, other viral promoters might also be similarly regulated. However, before this could be investigated, it was decided that a MIEP-specific RTQ-PCR should be devised to ensure more quantitative analyses of ChIP samples.

6.2 Results

6.2.1 Development of MIEP-specific RTQ-PCR analysis

A set of four PCR primers was designed such that they could be used for analysis of viral DNA from any strain of HCMV. Additionally, primers were designed to anneal between the sites of primers already used for all the semi-quantitative analysis carried out so far: +13 to -272 relative to the transcription start site (Fig. 6.1). Both sense and anti-sense primers were first tested in all combinations, using previously isolated HCMV DNA, at $MgCl_2$ concentrations of 2.5 and 3.5mM and SYBR Green reagent (for a full description of the method, see section 2.8.2.2). RTQ-PCR reactions were then analysed using a Rotor-Gene 3000 real-time PCR machine with a graphical representation of the data presented in Fig. 6.2. All combinations of primers produced positive reactions with both $MgCl_2$ concentrations used. However, the combination of sense primer S and anti-sense primer A (Fig. 6.2, blue lines) produced data with the lowest cycle threshold (C_t), which is inversely proportional to the efficiency of PCR. Indeed, this result was not surprising as the primer

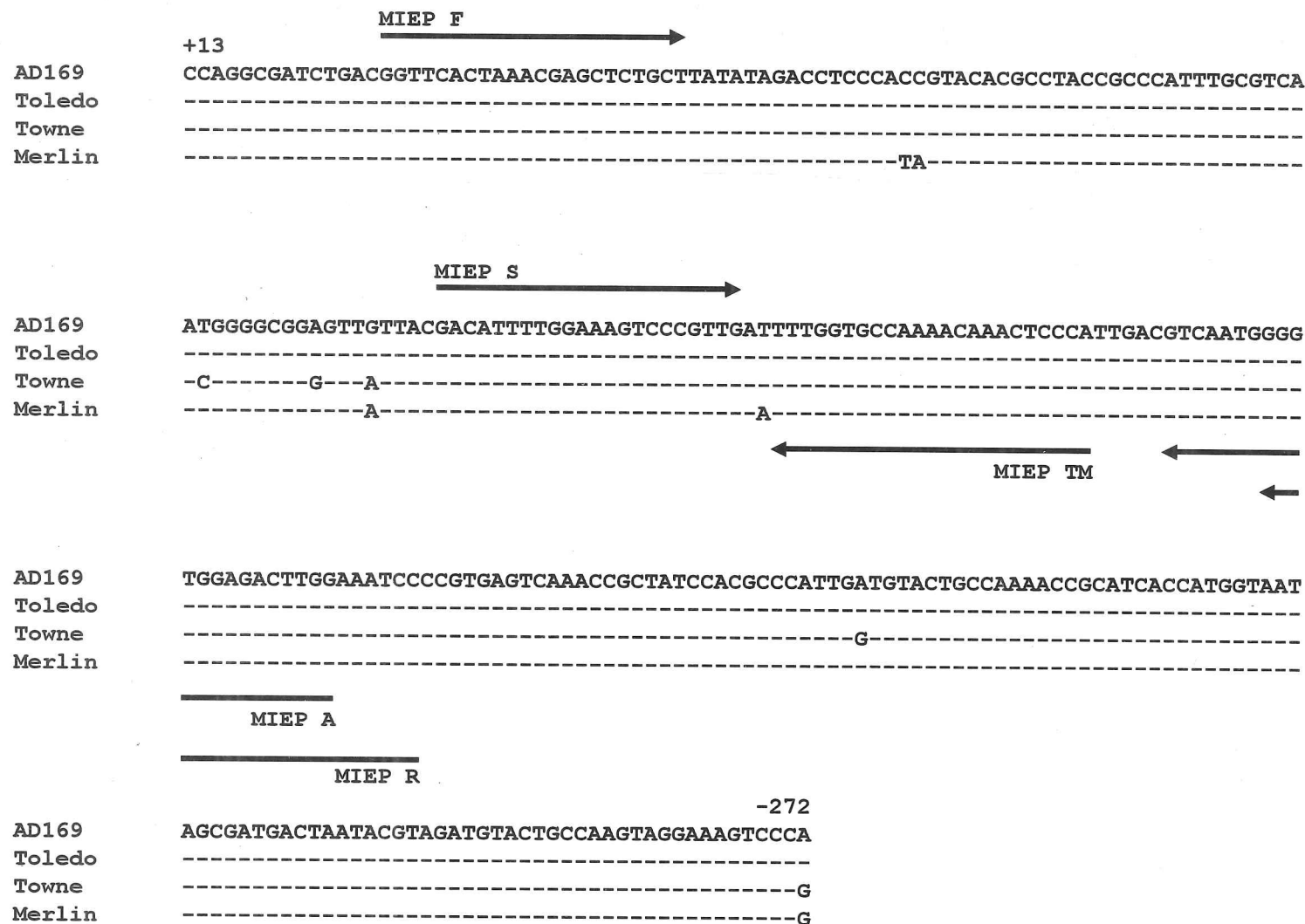


Figure 6.1 MIEP RTQ-PCR primer sites. The figure shows the sites of four primers, sense (S), antisense (A), forward (F) and reverse (R) and the TaqMan probe (TM) for the MIEP specific RTQ-PCR analysis positioned along the aligned MIEP sequences of four strains (AD169, Toledo, Towne and Merlin) of HCMV from +13 to -272 relative to the MIEP transcription start site.

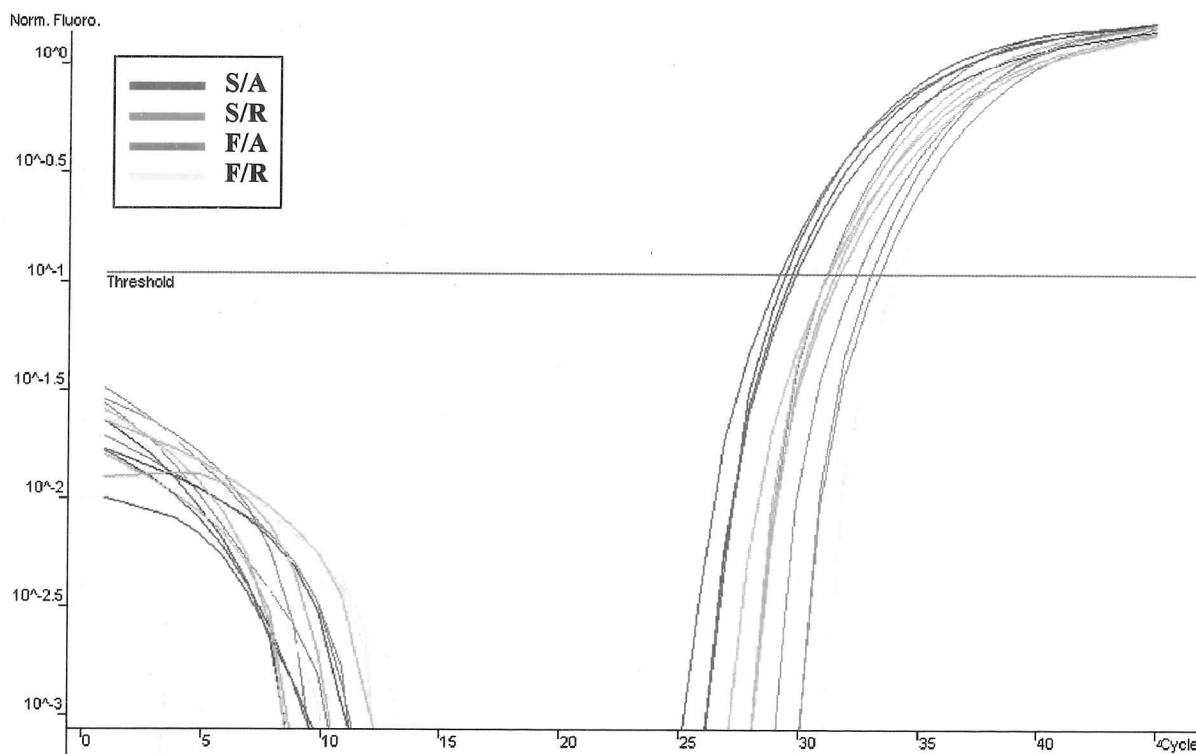


Figure 6.2 Optimisation of primer combination for MIEP-specific RTQ-PCR. PCR reactions were set up in duplicate using SYBR Green such that MIEP-specific sense primers (S & F) and antisense primers (A & R) were each used in combination at a MgCl_2 concentration of 2.5 & 3.5mM. Above is a graphical representation of the data. Primer combinations: S/A, blue; S/R, green; F/A, red; F/R, yellow.

pair highlighted produced the smallest PCR product (Fig. 6.1), which is generally accepted to promote efficient RTQ-PCR reactions.

It was next necessary to determine the optimum concentration of primers and TaqMan probe used for TaqMan MIEP-specific RTQ-PCR (for a full description of TaqMan RTQ-PCR, see section 2.8.2.1). The volume of primers S and A were varied from 0.1 to 1.8 μ l per reaction whilst the TaqMan probe concentration was varied from 0.05 to 0.2 μ l (see Table 6.1). RTQ-PCR was then performed on isolated HCMV DNA and the data is represented graphically in Fig. 6.3. TaqMan RTQ-PCR produced positive reactions at all concentration combinations, which are grouped together closely. In order to differentiate between the closely grouped data, the reactions were assessed through the C_t value (Table 6.1). Although combinations of primers and probe produced a C_t value as low as 15.54 (Table 6.1, U), the combination that produced the lowest C_t value with the least TaqMan probe used was J (primer S - 0.6 μ l; primer A - 0.1 μ l; TaqMan probe - 0.05 μ l). The reaction curve produced by combination J is also highlighted in Fig. 6.3. Therefore, this combination of primers and TaqMan probe was used routinely in all further experiments.

6.2.2 MIEP-specific RTQ-PCR can be employed for analysis of ChIP samples

The optimised combination of primers and TaqMan probe, established for the MIEP-specific RTQ-PCR was carried out using isolated HCMV DNA. However, it had not yet been tested on ChIP assay samples. Therefore, fibroblasts were infected (MOI 0.1) and ChIP analysis was carried out as before (Chapter 3). Resultant ChIP samples were then analysed by MIEP-specific RTQ-PCR for the level of association of modified histones and histone-associated proteins with viral MIEPs. The raw data produced and PCR curves are represented in Fig. 6.4a. In addition to RTQ-PCR of ChIP samples, for full quantification of data standard sample RTQ-PCRs were simultaneously carried out with known copy number dilutions of plasmid containing the MIEP (pEScat). An example of this standard curve is presented in Fig. 6.4b. Triplicate PCRs of standard plasmids can be seen as closely associated blue dots on the curve, whilst samples are represented along the curve (red dots). Sample data, normalised to the standard curve, was then calculated by the Rotor-Gene software and a threshold level set at which quantitative results could be determined within the linear range of PCR (Fig. 6.5). Numerical data is presented as a chart with low

	Primer S (μ l)	Primer A (μ l)	MIEP Taqman probe (μ l)	<i>Ct</i> ave
A	0.1	0.1	0.05	16.09
B	0.1	0.1	0.1	15.85
C	0.1	0.1	0.2	15.84
D	0.1	0.6	0.05	18.16
E	0.1	0.6	0.1	17.59
F	0.1	0.6	0.2	17.30
G	0.1	1.8	0.05	18.75
H	0.1	1.8	0.1	18.48
I	0.1	1.8	0.2	18.26
J	0.6	0.1	0.05	15.94
K	0.6	0.1	0.1	15.77
L	0.6	0.1	0.2	15.80
M	0.6	0.6	0.05	17.81
N	0.6	0.6	0.1	17.40
O	0.6	0.6	0.2	16.95
P	0.6	1.8	0.05	18.42
Q	0.6	1.8	0.1	18.00
R	0.6	1.8	0.2	17.77
S	1.8	0.1	0.05	16.04
T	1.8	0.1	0.1	15.64
U	1.8	0.1	0.2	15.54
V	1.8	0.6	0.05	17.45
W	1.8	0.6	0.1	17.24
X	1.8	0.6	0.2	16.99
Y	1.8	1.8	0.05	18.07
Z	1.8	1.8	0.1	17.95
α	1.8	1.8	0.2	17.60

Table 6.1 Optimisation of primers and TaqMan probe for MIEP-specific RTQ-PCR. PCR reactions were set up in triplicate such that MIEP-specific primers (S & A) and TaqMan probe were each titrated across a range of volumes: 0.1 to 1.8 μ l for primers and 0.05 to 0.2 μ l for TaqMan probe. The resultant average *Ct* (cycle threshold) value for each reaction is presented above. **Bold** = lowest *Ct* value with the lowest volume of TaqMan probe used.

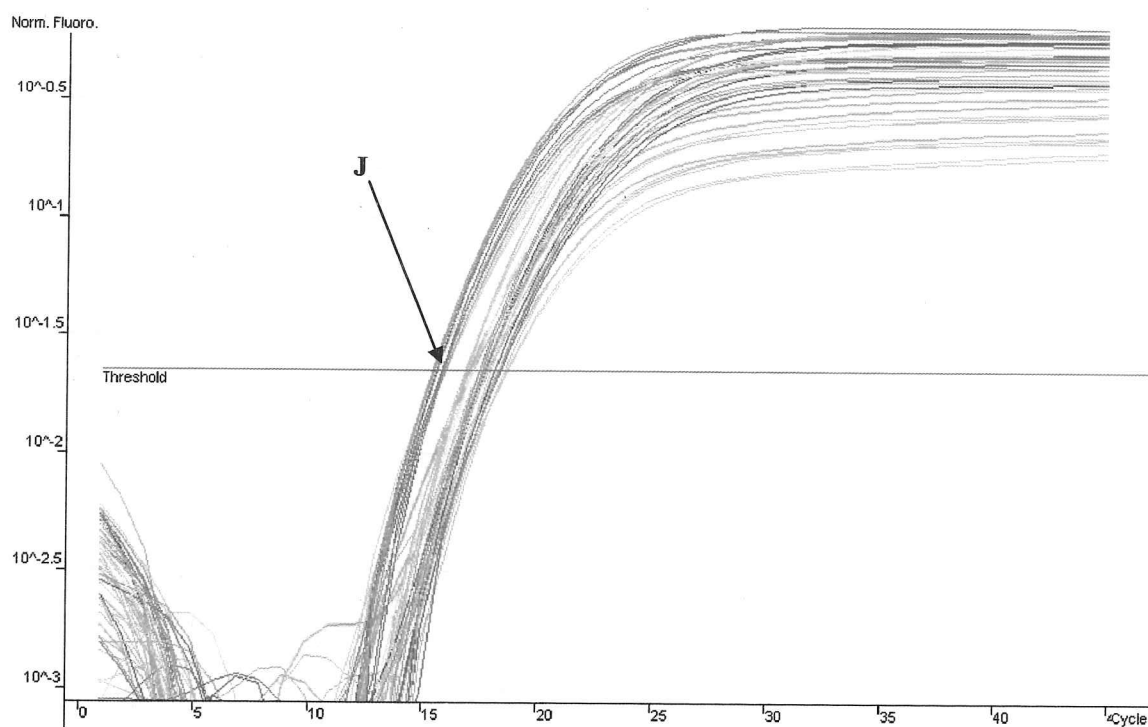
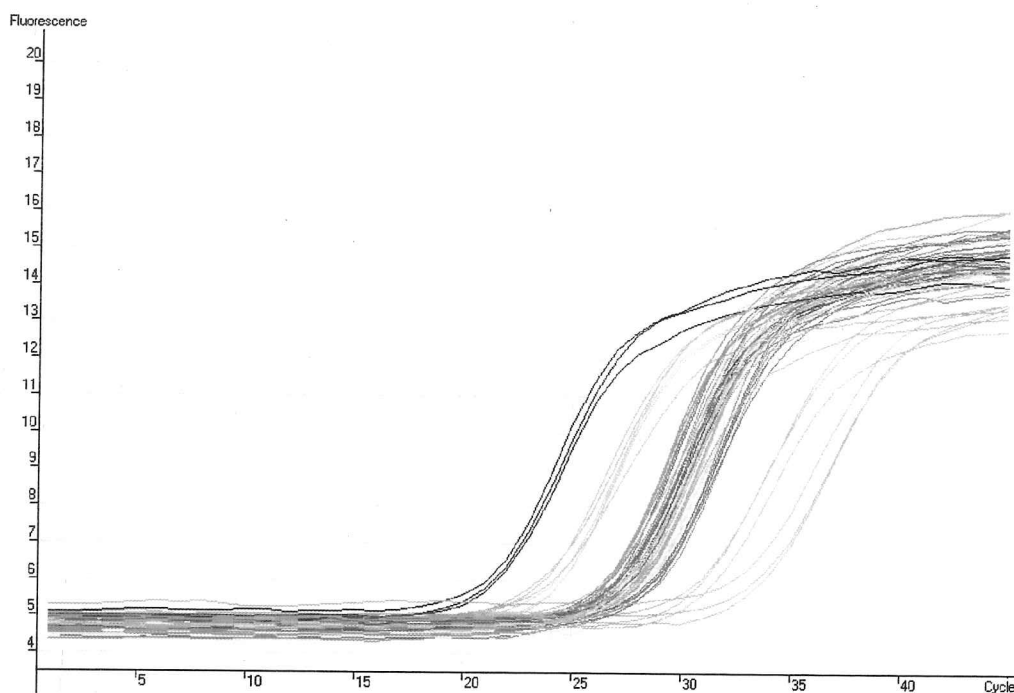


Figure 6.3 Optimisation of primers and TaqMan probe for MIEP-specific RTQ-PCR. PCR reactions were set up in triplicate such that MIEP-specific primers (S & A) and TaqMan probe were each titrated across a range of volumes: 0.1 to 1.8 μ l for primers and 0.05 to 0.2 μ l for TaqMan probe. Above is a graphical representation of the data presented in Table 6.1. (J) the highest C_t value with the lowest volume of TaqMan probe used per reaction.

A



B

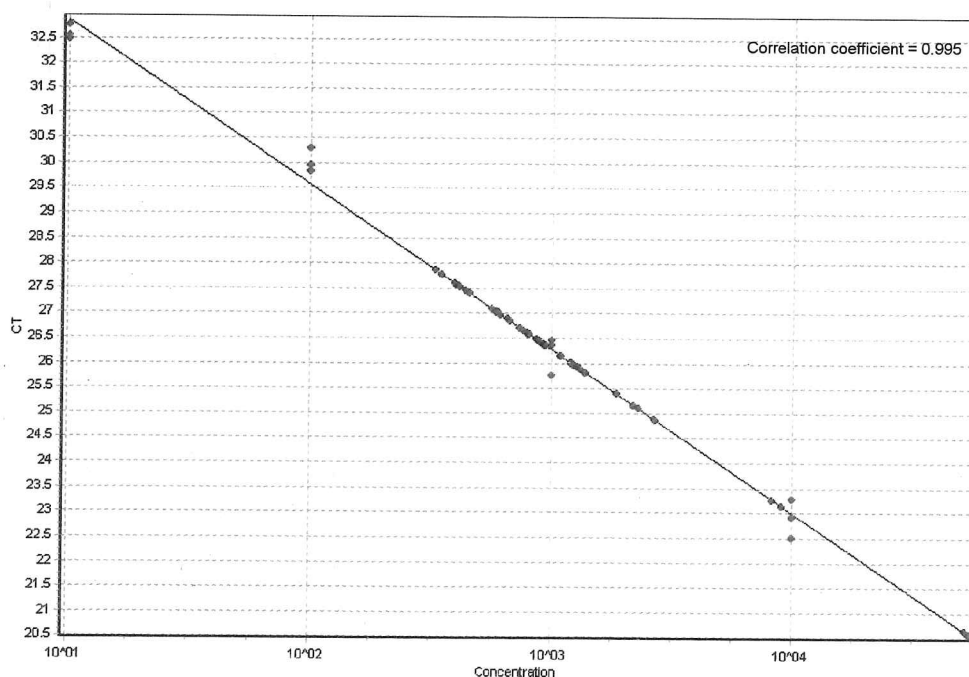


Figure 6.4 MIEP-specific RTQ-PCR of 3-96 hours post-infection ChIP analysis samples: raw data and standard curve. PCR reactions were set up in triplicate for ChIP samples produced 3-96 hours post-infection. (A) Graphical representation of raw data produced by TaqMan RTQ-PCR. (B) Graphical representation of the standard curve for the RTQ-PCR run using triplicate reactions (blue dots). Samples are shown along the standard curve (red dots).

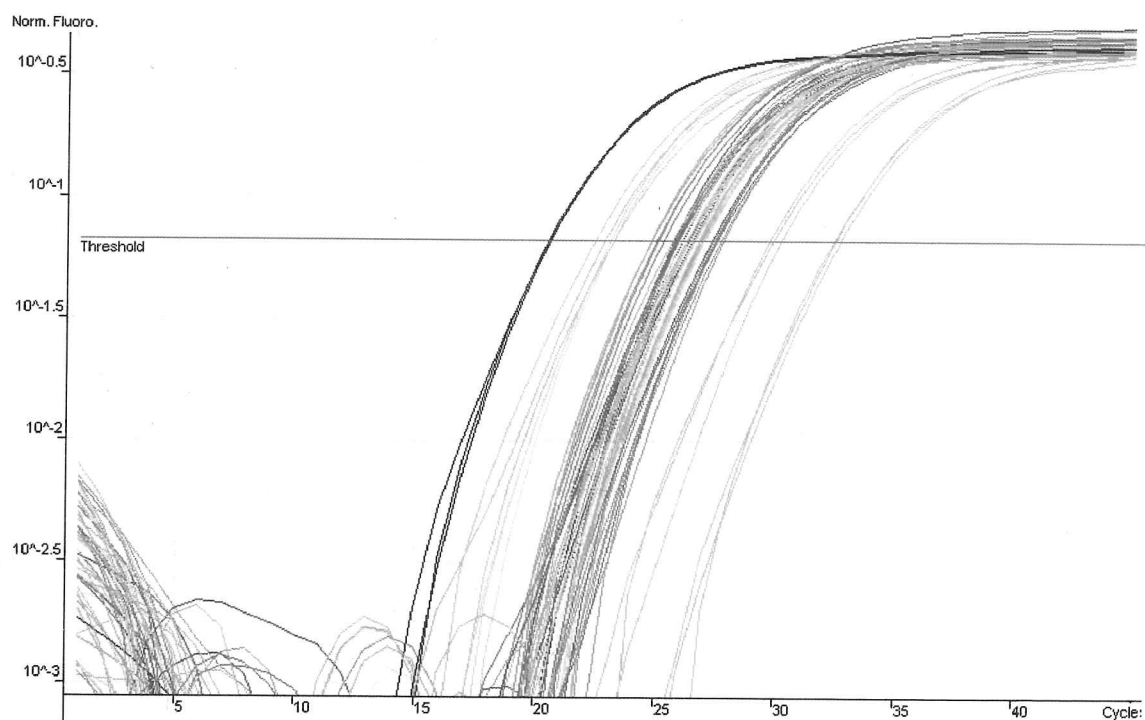


Figure 6.5 MIEP-specific RTQ-PCR of 3-96 hours post-infection ChIP analysis samples: compensated data. Graphical representation of PCR curves after standard curve has been applied to data for 3-96 hours post-infection ChIP assay samples. Threshold line (red) at which C_t value is taken and copy number calculated is shown.

level serum-control background subtracted from the sample data and results normalised to inputs - given a arbitrary value of 1.0 (Fig. 6.6).

At 3 hours post-infection, although the viral MIEP was associated with some acetylated histone-H4 (Fig. 6.6a, lane 1) and H3K9me2 (Fig. 6.6a, lane 2), it was predominantly associated with HP1 β (Fig. 6.6a, lane 3), consistent with previous semi-quantitative analysis (Chapter 3, Fig. 3.2). These quantitative data again corresponded with levels of IE gene transcription driven by the MIEP at this time. At 24 hours post-infection, the proportion of viral MIEPs associated with acetylated histones increased (Fig. 6.6b, lane 1) approximately 20-fold compared to 3 hours post-infection. In addition, there was also an increase in the association of H3K9me2 onto the MIEP (Fig. 6.6b, lane 2). Interestingly, although at this early timepoint major IE gene expression is still elevated, a recruitment of HP1 β could be seen (Fig. 6.6b, lane 3). Nevertheless, the MIEP remains predominantly associated with acetylated histone-H4 at this time. By 96 hours post-infection, the recruitment of acetylated histone-H4 (Fig. 6.6c, lane 1) seen at 24 hours post-infection was lost with more than a 4-fold reduction. There was a small decrease in H3K9me2 association (1.8-fold) (Fig. 6.6c, lane 2). However, HP1 β appeared most associated with the MIEP at this late timepoint (Fig. 6.6c, lane 3) with levels around 3 times higher than any other marker, consistent with transcriptional repression of the MIEP at this timepoint.

In previous chapters, the c-Fos promoter had been used as a positive control for semi-quantitative ChIP analysis. For RTQ-PCR analysis, other target promoters were used for positive controls of immunoprecipitation. It was necessary to verify that immunoprecipitation of both transcriptionally active and repressive chromatin had occurred consistently at each timepoint. Consequently, two targets were employed: the GAPDH promoter and the γ -globin promoter. GAPDH is a housekeeping enzyme employed in glycolysis within the cell and, as such, the promoter of the gene is routinely associated with transcriptionally active chromatin. Indeed, even super-infection of cells with HSV has shown no change to the levels of acetylated histone-H3 and H4 at the GAPDH promoter (Coleman *et al.*, 2008). Consequently, it is a robust target to analyse promoters associated with acetylated histone-H4. In contrast, the human γ -globin gene encodes the foetal haemoglobin protein that is not produced after gestation; hence the promoter is associated with transcriptionally repressed chromatin in differentiated cells

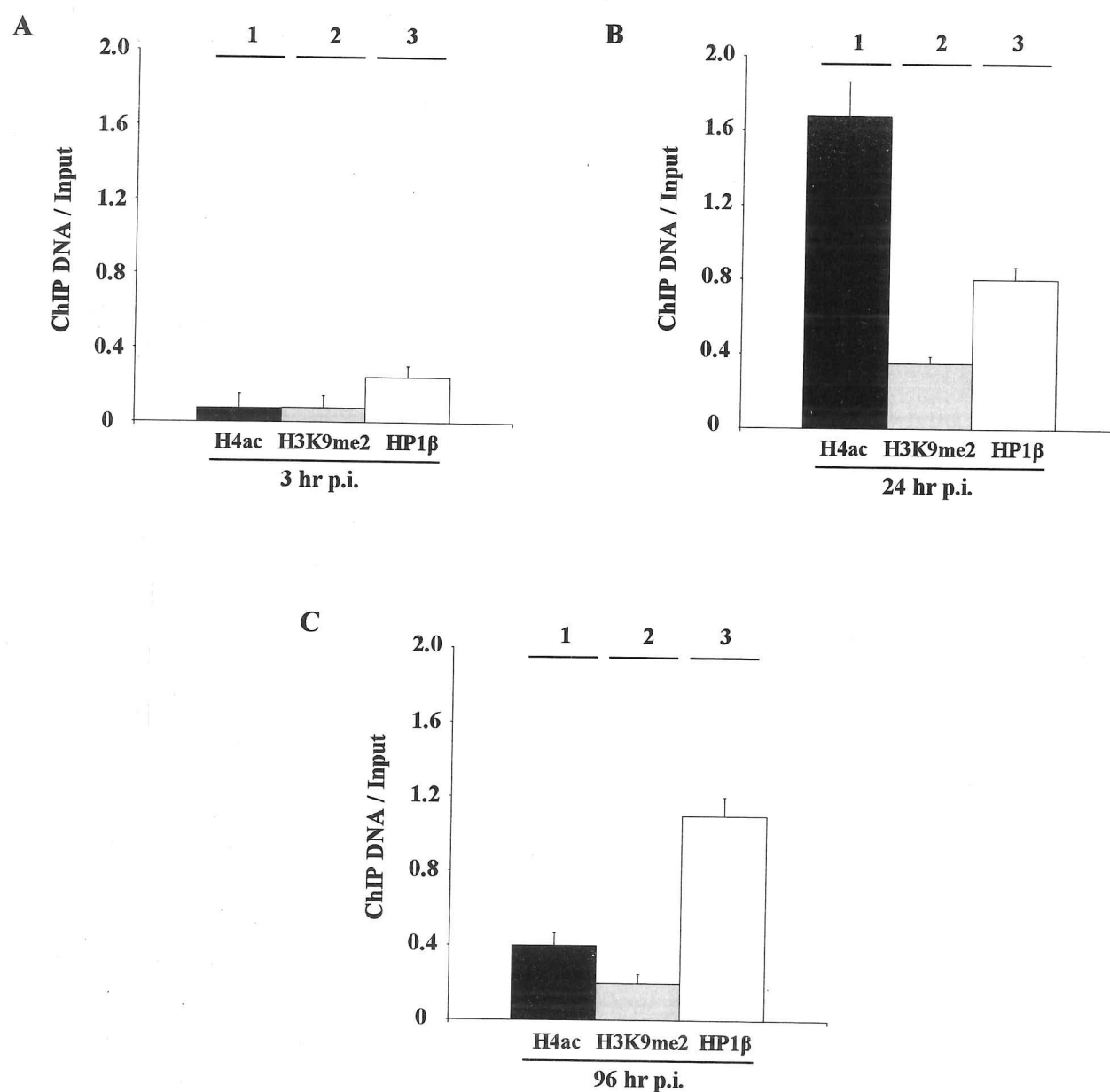


Figure 6.6 MIEP-specific RTQ-PCR can be employed for analysis of ChIP samples. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 24 and 96 (A-C) hours post-infection. Analysis was carried out with either an anti-acetylated histone-H4 antibody (lane 1), an anti-dimethyl lysine 9 histone-H3 antibody (lane 2) or an anti-HP1β antibody (lane 3). Isolated DNA was amplified using an MIEP-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

(Stamatoyannopoulos & Grosveld, 2001; Bottardi *et al.*, 2003). Additionally, the association of H3K9me2 and HP1 β with the γ -globin promoter is unaffected by herpesvirus infection (Reeves *et al.*, 2006; Coleman *et al.*, 2008). Therefore, it was routinely employed as a control for transcriptionally repressed chromatin association in all further experiments.

RTQ-PCR for the GAPDH promoter at the 3, 24 and 96 hour post-infection showed variable levels of association of the GAPDH promoter with acetylated histone-H4 (Fig. 6.7a). However, during the course of infection, the association with acetylated histone-H4 only varied up to two-fold (Fig. 6.7a, lanes 1 & 2). Consequently, this would have little effect on the overall profile of acetylated histone-H4 associated with the viral MIEP at each timepoint (Fig. 6.6). The data produced from γ -globin promoter RTQ-PCR for DNA immunoprecipitated with H3K9me2 (Fig. 6.7b) and HP1 β (Fig. 6.7c) again had an upper most range of two-fold change in efficiency: this level of variation would have little effect on the MIEP RTQ-PCR data for each antibody and timepoint illustrated in Fig 6.6. In addition, many studies normalise immunoprecipitation data directly to PCR from a single promoter, such as GAPDH, with only one antibody and one control target promoter. However, two RTQ-PCR targets were used here (GAPDH promoter and γ -globin promoter) due to the antibody targets. These controls generally show that the ChIP efficiency across the timescale of the experiment was consistent and robust.

6.2.3 *The MIEP is temporally regulated throughout productive infection by chromatin remodelling*

Thus far, the ChIP analyses had been carried out on permissive cells that had allowed full productive infection which includes viral DNA replication. Since it was possible that the ChIP results at later times of infection might have included newly synthesised and chromatinised MIEPs, phosphonoformic acid (PFA) was used to specifically inhibit viral DNA synthesis during infection (MOI 0.1) (see section 1.5.4). ChIP analysis was then carried out as in Fig. 6.6. Samples were analysed by MIEP-specific RTQ-PCR, including GAPDH promoter and γ -globin promoter RTQ-PCR to establish immunoprecipitation efficiency for each timepoint with each antibody.

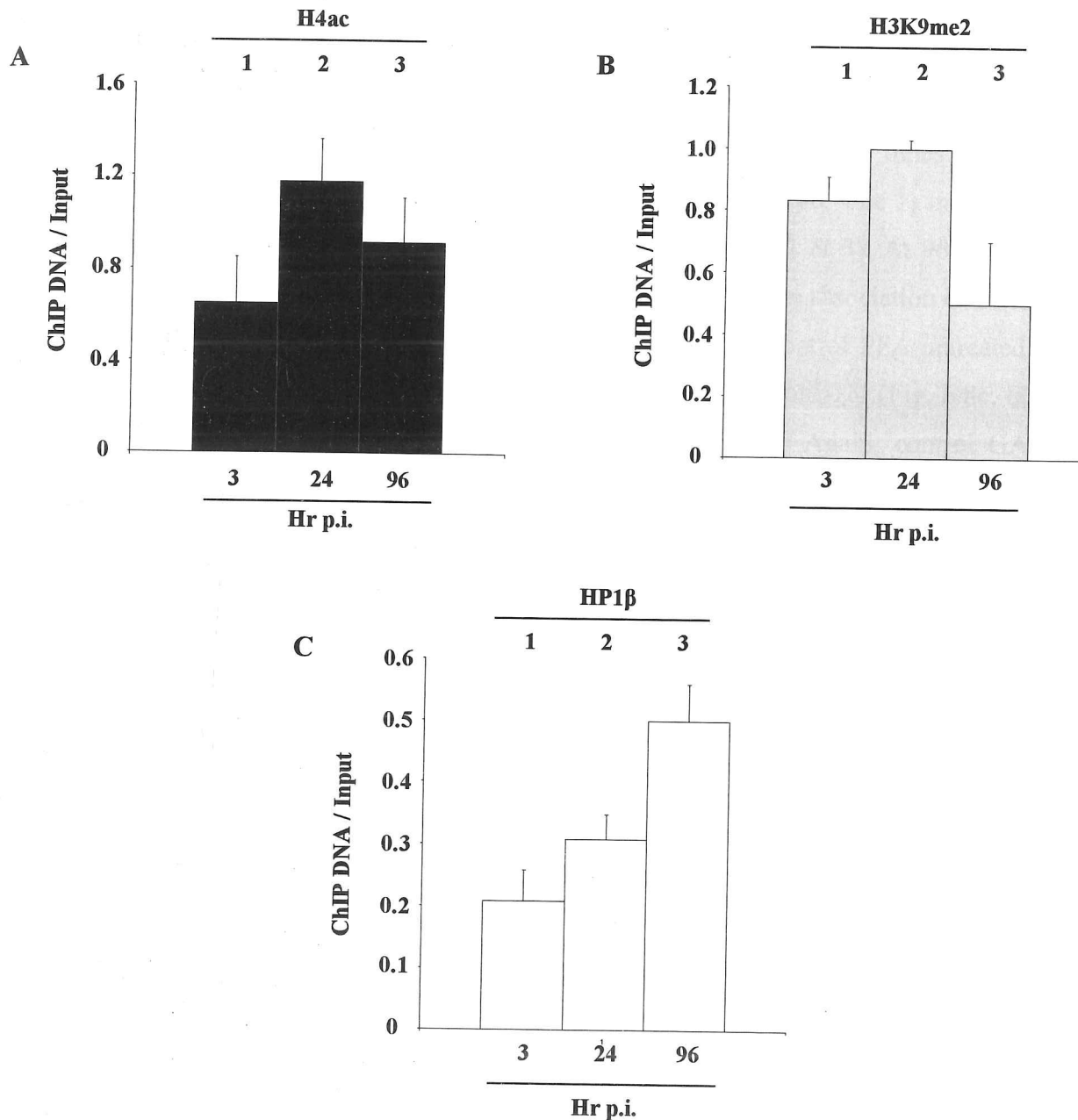


Figure 6.7 RTQ-PCR controls for chromatin immunoprecipitation. (A) ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 24 and 96 (lanes 1-3) hours post-infection. Analysis was carried out with an anti-acetylated histone-H4 antibody. Isolated DNA was amplified using a GAPDH-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. (B) Analysis was carried out with an anti-dimethyl lysine 9 histone-H3 antibody. Isolated DNA was amplified using a γ -globin-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. (C) Analysis was carried out with an anti-HP1 β antibody. Isolated DNA was amplified using a γ -globin-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of three independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

At 3 hours post-infection, the viral MIEP was associated with low levels of all forms of chromatin assessed by the study (Fig. 6.8a). The MIEP was associated with very low levels of acetylated histone-H4 (Fig. 6.8a, lane 1) but higher levels of HP1 β (Fig. 6.8a, lane 3), consistent with repression of IE gene transcription at this time. By early times of infection, acetylated histone-H4 recruitment had increased 24-fold (Fig. 6.8b, lane 1) in comparison to smaller increases of H3K9me2 and HP1 β (Fig. 6.8b, lanes 2 & 3). At 96 hours post-infection, in the absence of viral DNA replication due to PFA, the association of chromatin structure at the MIEP (Fig. 6.8c) actually appeared similar to that of PFA untreated cells (Fig. 6.6c) and showed a substantial decrease in acetylated histone-H4 (Fig. 6.8c, lane 1) with a considerable recruitment of HP1 β (Fig. 6.8c, lane 3). Again, control GAPDH promoter RTQ-PCR of acetylated histone-H4 immunoprecipitated samples (Fig. 6.9a) and γ -globin promoter RTQ-PCR of H3K9me2 and HP1 β immunoprecipitated samples (Fig. 6.9b & c respectively) showed less than 2-fold variation across the timecourse of the infection (Fig. 6.9b, lane 2) which could account for a slightly higher level of H3K9me2 than in other experiments on the MIEP at 24 hours post-infection. In order to confirm that PFA treatment had effectively inhibited viral DNA synthesis during infection, the MIEP-specific RTQ-PCR of input samples from untreated and PFA treated ChIP timecourses was analysed (Fig. 6.10). After correction for total DNA precipitation by GAPDH-specific RTQ-PCR, it can clearly be seen that when untreated fibroblasts are infected with HCMV Toledo the level of viral DNA increases 35-fold after 96 hours post-infection. In comparison, PFA treated fibroblasts show only a 2-fold increase in viral DNA level after 96 hours post-infection. Consequently, PFA treatment did efficiently prevent viral DNA replication and the small decrease in the amount of chromatin immunoprecipitated with the MIEP at late times of infection suggests that replicated viral genomes, or at least a proportion of them, do not become chromatinised.

6.2.4 An early promoter (UL44) is temporally regulated throughout productive infection by chromatin remodelling

Work presented in chapters 3 and 4 clearly demonstrated that the chromatin associated repression of viral IE gene expression immediately upon infection is mediated by chromatin remodelling via repressors and co-repressors, namely HDACs and hDaxx. The prevention of this repression by HDAC inhibitors and hDaxx down-regulation resulted in an increase of IE gene expression. However, it was also noted that relief of this repression

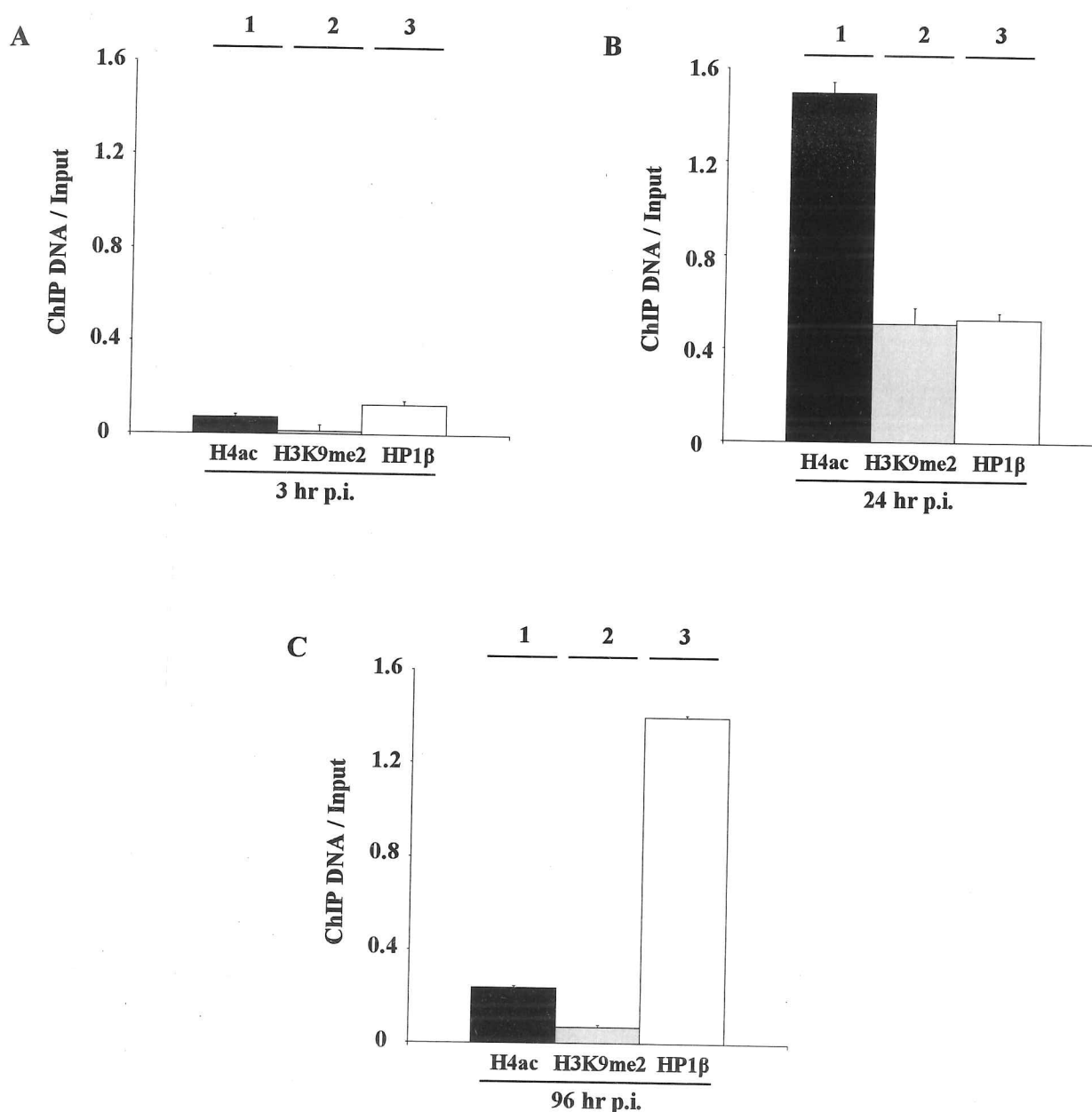


Figure 6.8 The MIEP of HCMV is chromatinised throughout a productive lytic infection – real-time quantitative-PCR. ChIP assays were performed (MOI 0.1) on serum-starved, PFA treated human fibroblasts at 3, 24 and 96 (A-C) hours post-infection. Analysis was carried out with either an anti-acetylated histone-H4 antibody (lane 1), an anti-dimethyl lysine 9 histone-H3 antibody (lane 2) or an anti-HP1β antibody (lane 3). Isolated DNA was amplified using an MIEP-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of two independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

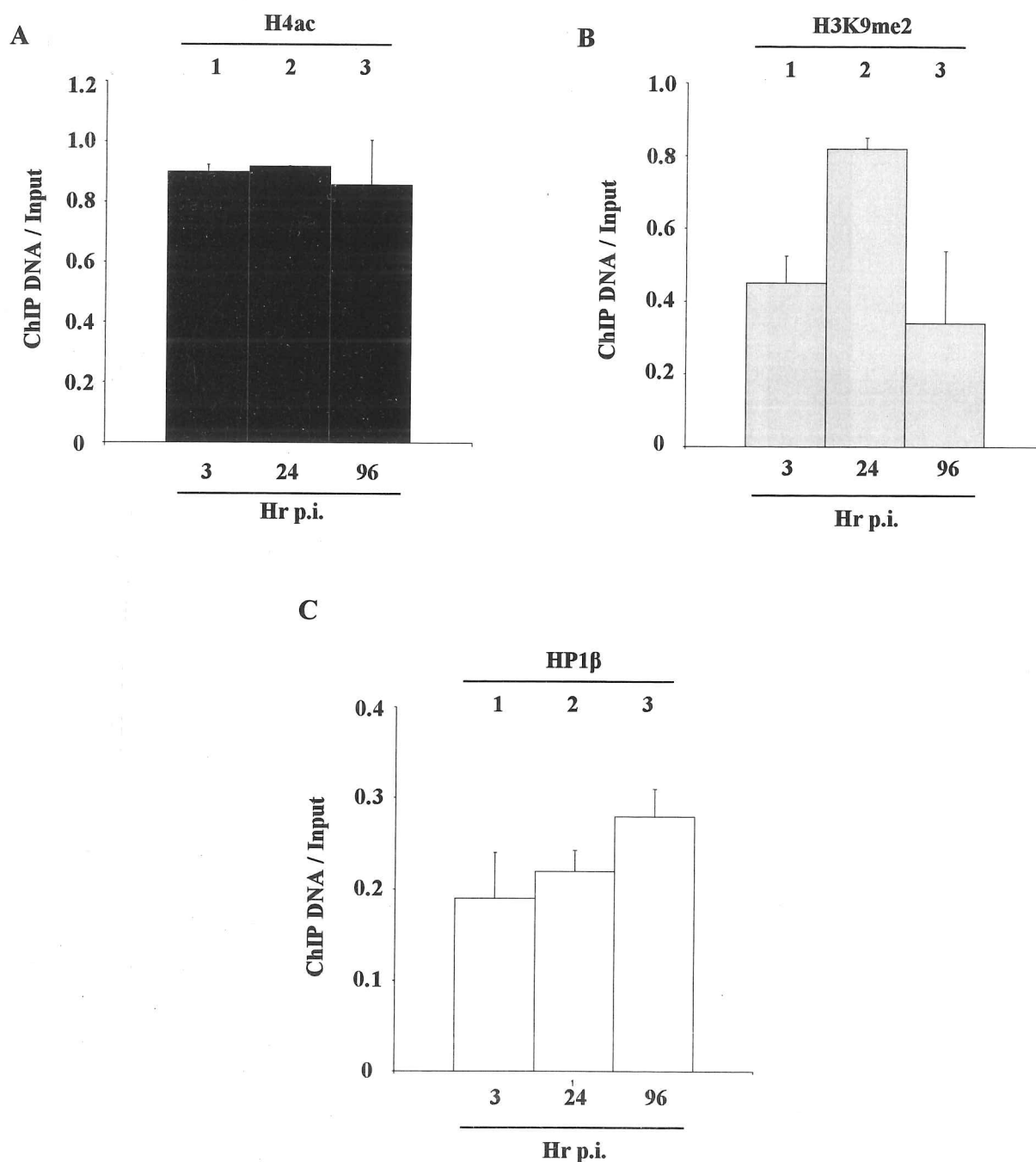


Figure 6.9 RTQ-PCR controls for chromatin immunoprecipitation. (A) ChIP assays were performed (MOI 0.1) on serum-starved, PFA treated human fibroblasts at 3, 24 and 96 (lanes 1-3) hours post-infection. Analysis was carried out with an anti-acetylated histone-H4 antibody. Isolated DNA was amplified using a GAPDH-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. (B) Analysis was carried out with an anti-dimethyl lysine 9 histone-H3 antibody. Isolated DNA was amplified using a γ -globin-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. (C) Analysis was carried out with an anti-HP1 β antibody. Isolated DNA was amplified using a γ -globin-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of two independent experiments. Error bars, 1 S.D. of triplicate PCR reactions.

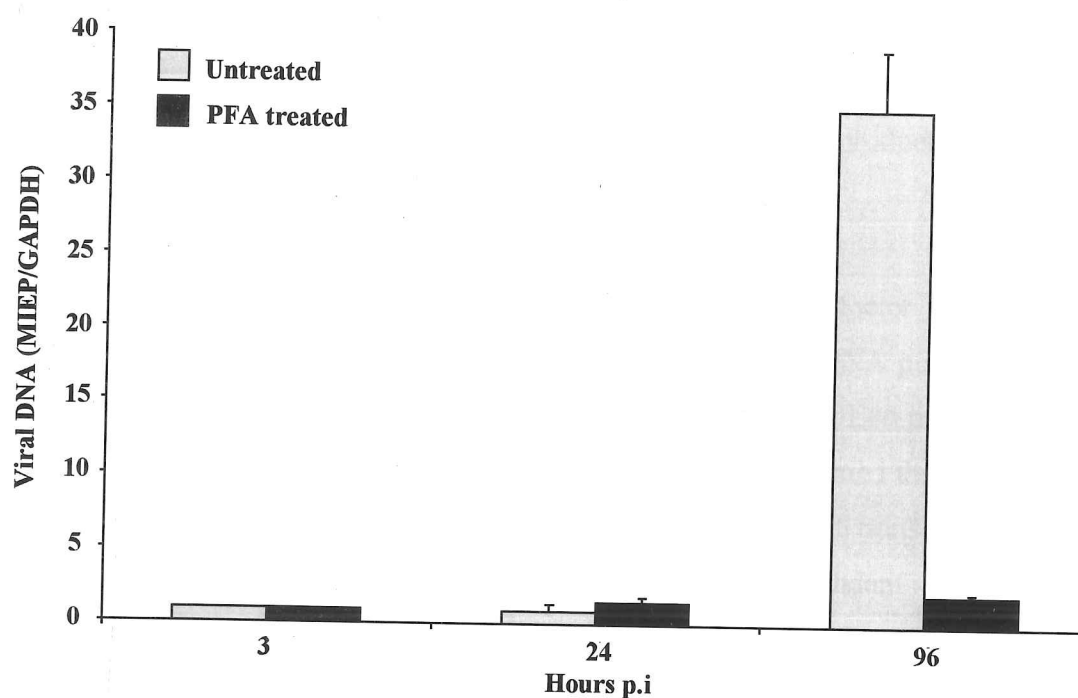


Figure 6.10 ChIP assay input data demonstrates inhibition of viral DNA synthesis with PFA treatment. ChIP assays were performed on either serum-starved, untreated (grey) or PFA treated (black) HCMV Toledo infected (MOI 0.1) human fibroblasts at 3, 24 and 96. Both MIEP and GAPDH RTQ-PCR was then performed on input samples and the quantity of viral DNA expressed as a proportion of the isolated total DNA (MIEP/GAPDH) relative to the 3 hour post-infection level (taken as 1.0). Error bars, 1 S.D. of three independent experiments.

additionally resulted in an increase of early and late viral gene expression. Since a recently published study at the time had shown an increasing association of acetylated histone-H4 at the promoter of the UL44 early gene over the first 12 hours of infection (Nevels *et al.*, 2004b), ChIP analysis of this promoter during the course of a full productive infection was next carried out.

The UL44 protein (p52) is a double-stranded DNA processivity factor required for viral DNA synthesis and is able to interact specifically with the viral DNA polymerase (UL54) (Pari *et al.*, 1993; Weiland *et al.*, 1994; Ripalti *et al.*, 1995). The UL44 promoter has three initiation sites: the distal and proximal initiate at early times, regulated through a canonical TATA sequence (TATAA; -163 & -58 from ATG). Initiation of transcription from the middle basal promoter occurs at late times of infection, dependent upon viral DNA synthesis and a non-canonical TATA sequence (-112 from ATG). The resulting gene product predominantly affects late viral gene expression rather than viral DNA synthesis (Leach & Mocarski, 1989; Isomura *et al.*, 2007; Isomura *et al.*, 2008b). As previous work had successfully utilised RTQ-PCR of the proximal UL44 promoter, with the PCR target positioned from -27 to +248 in relation to the ATG (Nevels *et al.*, 2004b), the same primer set was employed in this investigation. Furthermore, to provide a standard template that could be used for the production of a standard curve upon each RTQ-PCR run, the PCR product was first cloned into the pcDNA3 backbone plasmid before dilution to the appropriate concentrations and subsequent amplification. ChIP samples previously analysed by MIEP-specific RTQ-PCR (Fig. 6.6) were now analysed using UL44 promoter-specific RTQ-PCR (Fig. 6.11).

At 3 hours post-infection, the UL44 promoter was associated with low levels of modified histones (Fig. 6.11a). Although this comprised equal levels of both acetylated histone-H4 (Fig. 6.11a, lane 1) and HP1 β (Fig. 6.11a, lane 3), a 2.5-fold higher recruitment of the marker of transcriptional repression H3K9me2 was evident (Fig. 6.11a, lane 2) by this timepoint. This is consistent with the known lack of expression of UL44 at this time of infection from this proximal promoter. By 24 hours post-infection, though, the chromatin profile of the UL44 promoter had changed considerably (Fig. 6.11b). The proportion of UL44 promoters associated with H3K9me2 decreased by approximately 20% (Fig. 6.11b, lane 2) and the association of acetylated histone-H4 increased over 6.5-fold (Fig. 6.11b,

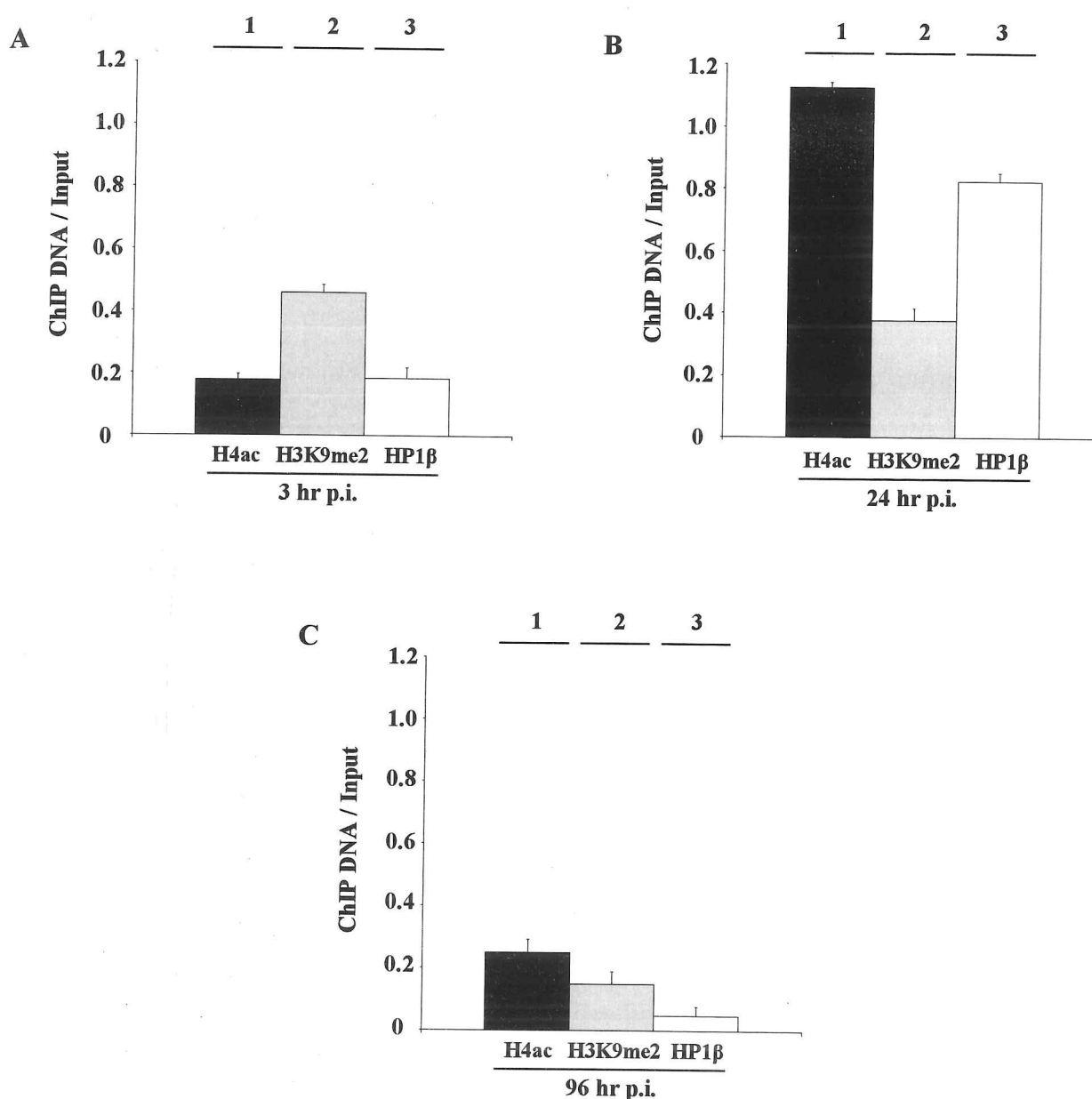


Figure 6.11 An early promoter (UL44) of HCMV is chromatinised throughout a productive lytic infection. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 24 and 96 (A-C) hours post-infection. Analysis was carried out with either an anti-acetylated histone-H4 antibody (lane 1), an anti-dimethyl lysine 9 histone-H3 antibody (lane 2) or an anti-HP1β antibody (lane 3). Isolated DNA was amplified using an UL44-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of two independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

lane 1) consistent with the initiation of early viral gene expression at this time. Intriguingly, there was also an increase in association of the promoter with HP1 β (Fig. 6.11b, lane 3). However, by late times of infection, this increase in HP1 β association seen at 24 hours post-infection was lost almost entirely (19-fold reduction) (Fig. 6.11c, lane 3). Indeed, all forms of associated histones appeared to decrease by this timepoint (Fig. 6.11c), although the promoter was still predominantly associated with acetylated histone-H4 (Fig. 6.11c, lane 1).

6.2.5 A late promoter (pp28) is temporally regulated throughout productive infection by chromatin remodelling

As chromatin remodelling of an early promoter throughout productive infection clearly occurred during infection and increased late gene expression also occurred with TSA treatment or hDaxx down-regulation in these cells, a late promoter was next analysed by ChIP analysis. To this end, the true late promoter of the tegument phosphoprotein pp28 was chosen. The pp28 region has at least two promoter elements: a true late promoter and an upstream promoter that has been shown to be up-regulated at early times by virus infection (Depto & Stenberg, 1992). True late gene expression is absolutely dependent upon viral DNA synthesis and is driven from -609 to +106 in relation to the transcriptional start site with leader sequences present from -6 to +46 regulating the translation of late transcripts (Kohler *et al.*, 1994; Kerry *et al.*, 1997). Therefore, primers were designed to anneal between the upstream promoter leader and the putative translational regulatory element, resulting in amplification of nucleotides from -290 to -180 of the true late region in relation to the transcriptional start site. Again, to act as standard controls for the RTQ-PCR, the PCR product was cloned into the pcDNA3 backbone plasmid before dilution to the appropriate concentrations and subsequent amplification. Analysis was then carried out on the 3-96 hour post-infection ChIP samples.

At 3 hours post-infection, the MIEP was predominantly associated with H3K9me2 (Fig. 6.12a, lane 2) with little association of acetylated histone-H4 (Fig. 6.12a, lane 1) or HP1 β (Fig. 6.12a, lane 3). By 24 hours post-infection, the profile of chromatin association at the pp28 promoter changed little: a 15% increase in H3K9me2 (Fig. 6.12b, lane 2) was concurrent with decreases in acetylated histone-H4 (2-fold) (Fig. 6.12b, lane 1) and HP1 β (2.5-fold) (Fig. 6.12b, lane 3). This is consistent with the known lack of transcription from

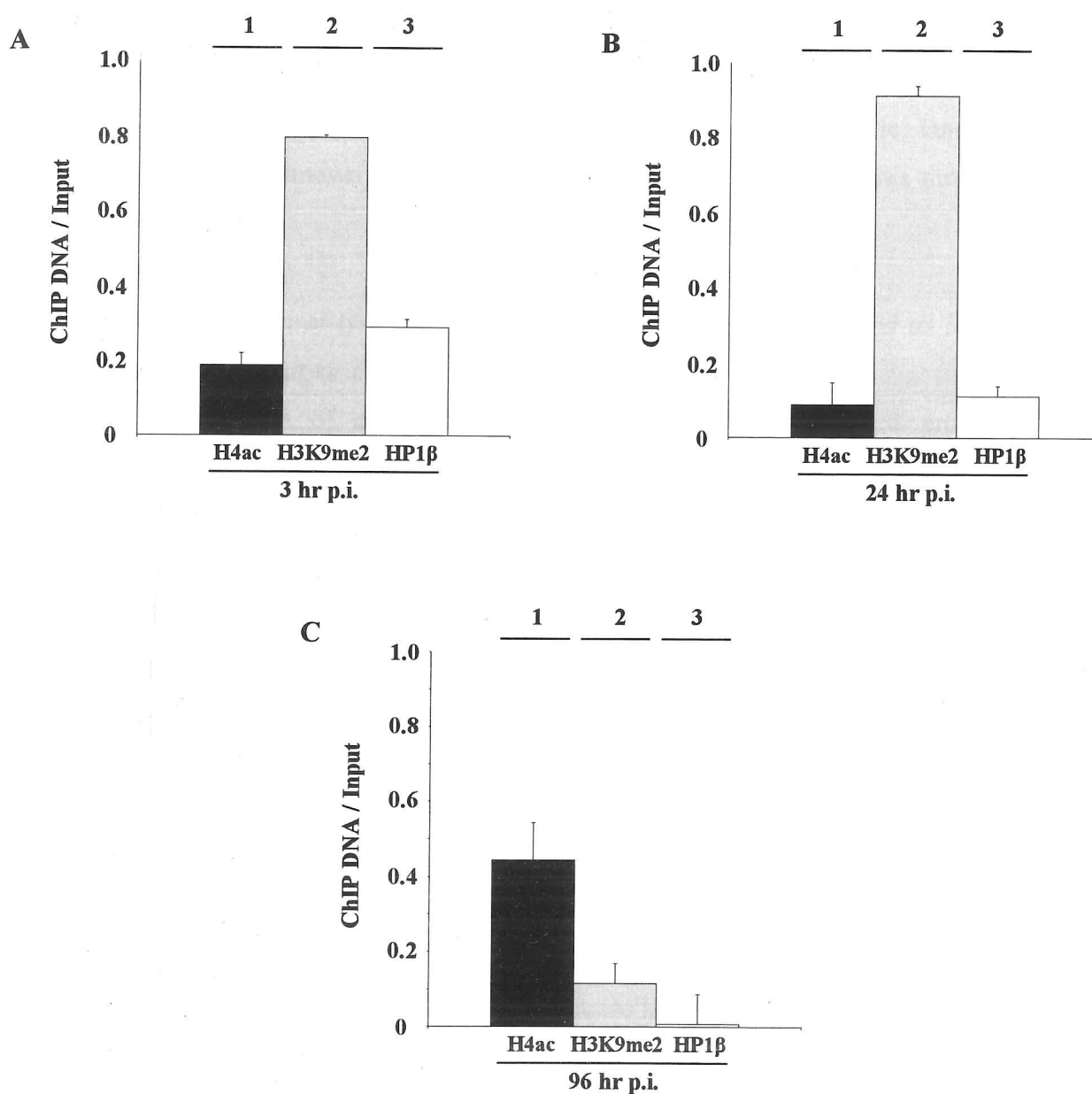


Figure 6.12 A late promoter (pp28) of HCMV is chromatinised throughout a productive lytic infection. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 24 and 96 (A-C) hours post-infection. Analysis was carried out with either an anti-acetylated histone-H4 antibody (lane 1), an anti-dimethyl lysine 9 histone-H3 antibody (lane 2) or an anti-HP1 β antibody (lane 3). Isolated DNA was amplified using an pp28-specific RTQ-PCR and for comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data is representative of two independent experiments. *Error bars*, 1 S.D. of triplicate PCR reactions.

this promoter at this time of infection. However, by 96 hours post-infection a significant change in the chromatin profile occurred: whilst HP1 β decreased 12-fold (Fig. 6.12c, lane 3) and H3K9me2 8-fold (Fig. 6.12c, lane 2), the promoter was predominantly associated at this timepoint with acetylated histone-H4 (a 5-fold increase) (Fig. 6.12c, lane 1). This is consistent with the known levels of transcription of this late gene at this timepoint post-infection.

6.2.6 Di-methylation of lysine 4 of histone-H3 (H3K4me2) can be used as a marker for active transcription at viral promoters

Immunoprecipitation of chromatin markers for active and repressed promoters had determined that all classes of HCMV genes were temporally associated with chromatin structure throughout productive infection, and that, in general, this reflected the known gene expression profiles from these promoters. However, other chromatin modifications have been characterised during the course of this work. Notably, whilst di-methylation of lysine 9 on histone-H3 (H3K9me2) is a known marker for repressive chromatin (Kouzarides, 2007a), di-methylation of lysine 4 on histone-H3 (H3K4me2) has been characterised as a marker of an active promoter and transcriptional elongation in mammals (Schneider *et al.*, 2004). Furthermore, this chromatin modification has been assessed during HSV-1 productive infection and found to be temporally associated with promoters of viral genes (ICP0, TK and VP16) during transcription and preferentially associated with the 5' end of transcribed viral genes (Kent *et al.*, 2004). Therefore, the association of this modified histone was assessed at the HCMV major IE, UL44 and pp28 promoters during a timecourse of infection.

Fibroblasts were infected as above (MOI 0.1). At the appropriate timepoint (3, 8, 24, 48 or 96 hours post-infection), the cells were fixed and ChIP assays carried out using either control serum or an anti-di-methyl lysine 4 histone-H3 (H3K4me2) antibody with 10% of the total chromatin fraction used as input for each timepoint. Resultant ChIP samples were then analysed by promoter-specific RTQ-PCR and, subsequently, GAPDH promoter RTQ-PCR to establish immunoprecipitation efficiency for each timepoint with each antibody. The results, normalised relative to input and GAPDH promoter immunoprecipitation levels, are presented in Fig. 6.13 to Fig. 6.15.

At 3 hours post-infection, the MIEP was clearly associated with H3K4me2 (Fig. 6.13). This association increased 2.3-fold by 8 hours and a further 2-fold by 24 hours. Thereafter, association of H3K4me2 with the MIEP decreased 5.6-fold by 48 hours before a further decrease of 3-fold by 96 hours. Overall, this is consistent with the known levels of major IE gene transcription at these timepoints. Association of H3K4me2 with the UL44 promoter was not clearly detected until 24 hours post-infection (Fig. 6.14). However, this association then increased dramatically by 48 hours (5-fold) before a further 1.5-fold increase by 96 hours post-infection. Although the increases seen from 8 to 48 hours are consistent with early gene transcription, the high level of H3K4me2 association at 96 hours is unexpected although possibly due to the presence of further distal promoters within the UL44 gene and consistent with previous ChIP analysis (Fig. 6.11c). Low levels of H3K4me2 were observed associated with the pp28 promoter from 8 to 24 hours post-infection, whereupon a small increase occurred by 48 hours (Fig. 6.15). A further 11-fold increase also occurred at 48 hours until, at 96 hours post-infection, this was maximal (Fig. 6.12). These observations are also consistent with pp28 transcription throughout the course of infection and previous ChIP analysis (Fig. 6.12). Intriguingly, a high level of H3K4me2 association with the pp28 promoter was observed at 3 hours post-infection (40% of the maximal 96 hour post-infection level). However, overall, levels of association of H3K4me2 with the HCMV promoters analysed correlate directly with the temporal regulation of gene expression from those promoters.

6.2.7. A substantial proportion of viral genomes are chromatinised during productive infection

Although ChIP analyses presented in all chapters of this study show an association of modified histones and histone-associated proteins with promoters of various temporal classes of HCMV genes, the proportion of the total population of viral genomes associated with histones is unknown. This is important, since if only a small proportion of viral genomes were associated with histones/histone-associated proteins, then the relevance of changes in the chromatin structure of this low proportion of incoming viral genomes could be questioned. Therefore, ChIP analysis using an antibody specific for histone-H3 was carried out. After isolation of immunoprecipitated DNA, MIEP-specific RTQ-PCR was employed to quantify the number of viral genomes associated with histone-H3, whilst GAPDH-specific RTQ-PCR was used to quantify the efficiency of histone-H3

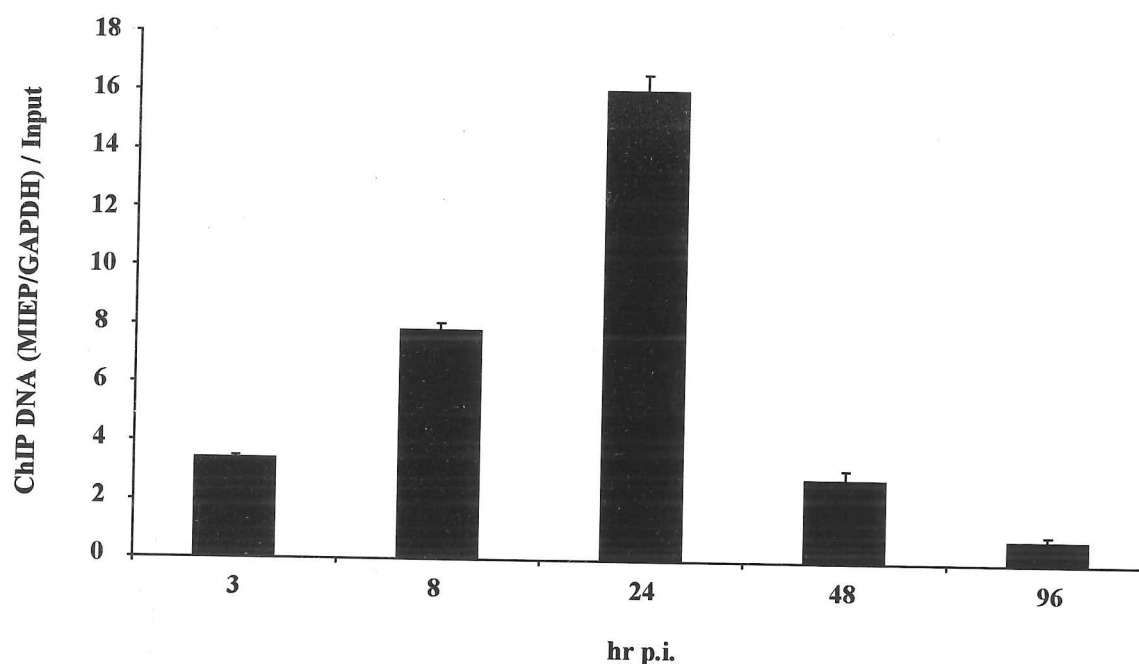


Figure 6.13 Di-methylation of lysine 4 histone-H3 (H3K4me2) acts as a marker for transcriptional expression at the MIEP. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 8, 24, 48 and 96 hours post-infection. Analysis was carried out with either serum control or an anti-H3K4me2 antibody. Isolated DNA was amplified using an MIEP-specific and a GAPDH-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA and the amount of MIEP DNA at each timepoint then normalised to the GAPDH loading control. The data is representative of two independent experiments. *Error bars*, 1 S.D. of triplicate MIEP PCR reactions.

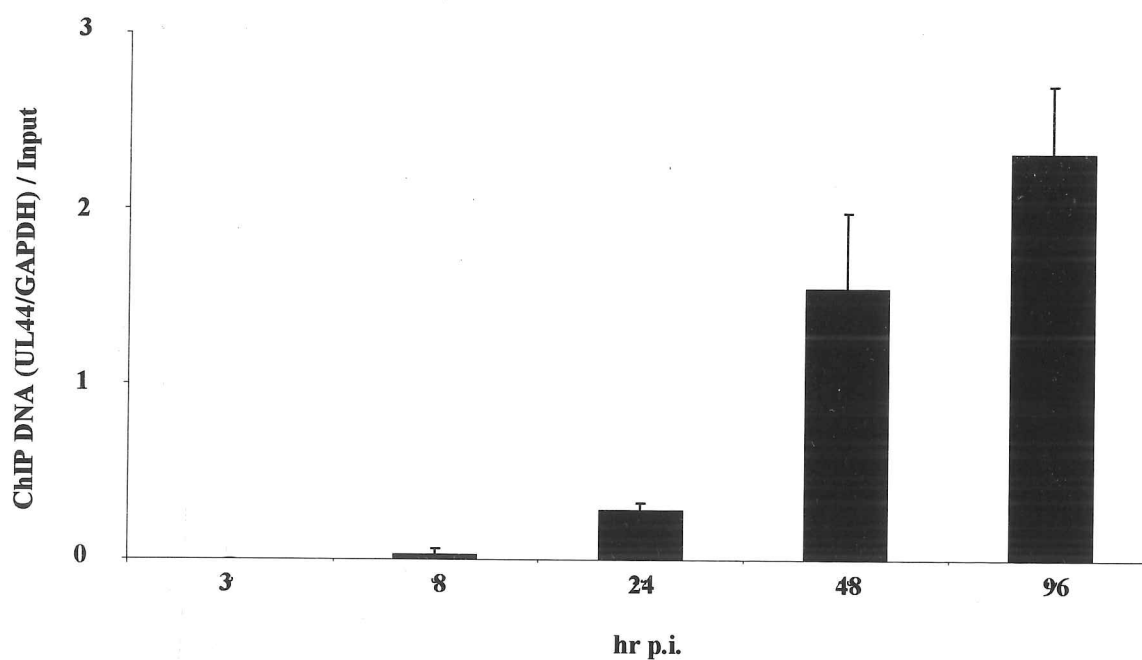


Figure 6.14 H3K4me2 acts as a marker for transcriptional expression at the UL44 promoter. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 8, 24, 48 and 96 hours post-infection. Analysis was carried out with either serum control or an anti-H3K4me2 antibody. Isolated DNA was amplified using an MIEP-specific and a GAPDH-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA and the amount of MIEP DNA at each timepoint then normalised to the GAPDH loading control. The data is representative of two independent experiments. *Error bars*, 1 S.D. of triplicate UL44 PCR reactions.

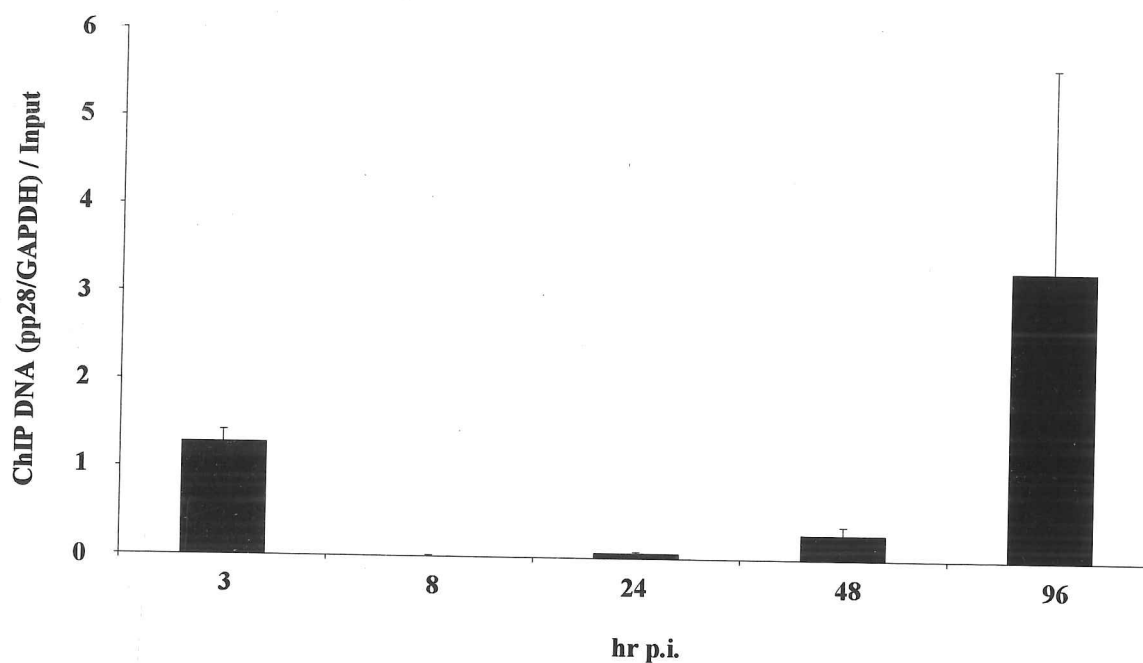


Figure 6.15 H3K4me2 acts as a marker for transcriptional expression at the pp28 promoter. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 3, 8, 24, 48 and 96 hours post-infection. Analysis was carried out with either serum control or an anti-H3K4me2 antibody. Isolated DNA was amplified using an MIEP-specific and a GAPDH-specific RTQ-PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA and the amount of MIEP DNA at each timepoint then normalised to the GAPDH loading control. The data is representative of two independent experiments. *Error bars*, 1 S.D. of triplicate pp28 PCR reactions.

immunoprecipitation. The number of viral genomes was then normalised using the efficiency of equivalent GAPDH promoter immunoprecipitation and the data is provided in table 6.2. At 8 hours post-infection, by which time histone-association has occurred with the viral genome, an average of 19.8% of virus genomes are associated with histone-H3. Thus, a substantial proportion of viral genomes are associated with histones at an IE timepoint of infection. However, it was not known whether, after viral DNA replication, newly synthesised genomes become associated with histones. Therefore, ChIP analysis for global histone-H3 was also carried out at 72 hours post-infection, by which time viral DNA synthesis would have occurred and samples were again assessed using RTQ-PCR analysis. At 72 hours post-infection, an average of 10.8% of viral genomes were associated with histone-H3, a decrease in the proportion of total genomes histone-associated. Although the range of results at this timepoint produced a higher standard error (6.6%), the 2-fold decrease in proportion of total genomes associated with histones is consistent with the decrease seen in total modified histone association of the MIE, UL44 and pp28 promoters at late times of infection.

6.3 Discussion

Control of viral major IE gene expression has been shown to be controlled by temporal association of chromatin structure at the MIEP throughout HCMV productive infection (see Chapter 3). In addition, relief from mediators of repression (HDACs – Chapter 3; hDaxx – Chapter 4) results in an increase in levels of expression of IE genes and an advance of the infection cycle such that higher levels of viral early and late gene expression also occur but earlier during infection. Recently, it has been shown that the promoters of all classes of HSV-1 genes are associated with histones throughout productive, lytic infection and modification of these histones occurs throughout infection in a temporally controlled manner (Herrera & Triezenberg, 2004; Kent *et al.*, 2004; Coleman *et al.*, 2008). Consequently, this chapter analysed the possibility that the promoters of all classes of HCMV genes (IE, early and late) were similarly modified during the timecourse of infection.

The establishment of real-time quantitative PCR (RTQ-PCR) for analysis of ChIP samples proved advantageous. It shortened the analysis time and removed the use of radioactivity to analyse samples, providing normalised, quantitative data. Analysis of the viral MIEP

<i>Experiment</i>	<i>% of total viral genomes immunoprecipitated</i>	
	<i>8 hr p.i.</i>	<i>72 hr p.i.</i>
1	19.1	17.1
2	19.7	11.4
3	20.6	4.0
Average (± 1 SD)	19.8 (± 0.8)	10.8 (± 6.6)

Table 6.2 A substantial proportion of viral genomes are chromatinised throughout productive infection. ChIP assays were performed (MOI 0.1) on serum-starved human fibroblasts at 8 and 72 hours post-infection. Immunoprecipitation was carried out with a pan-histone-H3 antibody. Isolated DNA was amplified using MIEP-specific RTQ-PCR and the immunoprecipitation efficiency in each sample normalised using GAPDH promoter-specific RTQ-PCR. The data represents three independent experiments.

throughout full productive infection confirmed the temporal association of chromatin structure seen in Chapter 3 consistent with the known levels of major IE gene transcription during infection. Analysis of a further marker of active transcription, H3K4me2, was also consistent as before. Indeed, such changes in the association of chromatin markers with the MIEP are also consistent with other analysis from our laboratory examining MIEP auto-regulation by IE86 (Reeves *et al.*, 2006). In part, this analysis (Reeves *et al.*, 2006) goes some way to explain the increased association of the MIEP with markers of transcriptional repression as the virus infection progresses. In addition, the temporal association of acetylated histone-H4 shown in Chapter 3 and in this chapter is consistent with recently published data (Park *et al.*, 2007b). However, RTQ-PCR analysis of ChIP samples showed a lower total association of modified histones with the MIEP at 3 hours post-infection than the semi-quantitative PCR analysis in Chapter 3. This is likely due to the specificity of RTQ-PCR, which provides data from the linear phase of amplification, and provides evidence that the MIEP is still undergoing recruitment of histones and histone associated proteins at this time of infection.

Other studies have also recently shown the association of early HCMV promoters with modified histones. The promoter of the UL44 gene has been shown to become associated with acetylated histones by 12 hours post-infection (Nevels *et al.*, 2004b) whilst the promoter of the viral DNA polymerase, UL54, becomes associated with acetylated histone-H4 by 48 hours post-infection (Park *et al.*, 2007b). The data presented in this chapter is consistent with these findings: at IE times the UL44 promoter was shown to be associated with H3K9me2 predominantly at an IE timepoint whereas by 24 hours post-infection the promoter had become predominantly associated with acetylated histone-H4, consistent with the known time course of UL44 transcription. However, and slightly surprisingly, at late timepoints, when UL44 expression is not expected, the promoter still appears associated with acetylated histone-H4 and H3K4me2. This may be consistent with the activation of a known upstream cryptic element in the UL44 promoter which is active at late times (Leach & Mocarski, 1989) and could cause the true promoter to appear to remain transcriptionally active. In addition, although it is known that factors, such as IE86, are responsible for negative auto-regulation of the MIEP (Reeves *et al.*, 2006), it is not known if such directed regulation of early or late viral promoters takes place or is necessary. Elucidation of this will require further work.

Chromatinisation of the pp28 true late promoter also occurred throughout infection. Again, the association of acetylated histone-H4 and H3K4me2 was consistent with the known profile of pp28 transcription during infection. However, at IE and early times of infection, when pp28 transcription is known to be low (Mocarski *et al.*, 2007), very little HP1 β was associated with the promoter. Instead, H3K9me2 was the predominantly associated factor. Therefore, it is possible that this 'H3K9me2-repressed' promoter state may be akin to a poised state that can become remodelled to an active state when the appropriate levels of viral factors (IE72 and IE86) become available. Interestingly, this association of H3K9me2 at IE times of infection was also observed with the early UL44 promoter.

Analysis of the pp28 promoter did show an association with H3K4me2, a marker of transcriptional activation, at 3 hours post-infection. Similar to the UL44 promoter, the pp28 promoter also contains certain elements that have been shown to be active at times of infection other than late (Depto & Stenberg, 1992). However, whether the activity of a cryptic element may be the cause of H3K4me2 association at this timepoint is unclear.

One consistent finding was that, at 96 hours post-infection, all viral promoters appeared less associated with known chromatin markers. This would be consistent with a dilution effect after viral DNA synthesis if newly replicated genomes were not chromatinised, as hypothesised with HSV-1 (Oh & Fraser, 2008). Indeed, although blocking of viral DNA synthesis by phosphonoformic acid did not change the association of markers of transcriptionally active or repressed chromatin at the MIEP, it did lead to a slight increase in total precipitated chromatin compared to untreated cells at late times on infection. This is consistent with the hypothesis that newly replicated viral genomes are less chromatinised. Furthermore, ChIP analysis of the association of histone-H3 with the MIEP demonstrated that although around 20% of viral genomes are associated with histones at 8 hours post-infection, by late times after viral DNA synthesis this proportion decreases 2-fold. This finding is consistent with the decrease of total modified histones associated with all viral promoters analysed and a dilution effect at late times of infection. However, although the proportion of viral genomes associated with histones decreases, the total number of viral genomes present increases after viral DNA synthesis and, as such, the number of genomes that are histone-associated increases. Whether this increase in histone association after replication aids repression of MIEP activity at late times of infection,

when IE gene transcription is not wanted, is unknown. However, ChIP analysis in the presence of PFA did not cause any particular change in the association of acetylated histone-H4 at late times of infection. Indeed, the increase in the total number of viral genomes present at late times and the decrease in histone association with this population would likely provide an environment conducive to packaging of viral DNA into capsids.

Overall, the data presented within this chapter show that the regulation of expression of all classes of HCMV genes during the full timecourse of virus infection involves chromatin remodelling of their respective promoters. The MIEP and promoters of prototypical early and late genes, UL44 and pp28 respectively, become associated with histones and histone associated proteins upon infection and the temporal regulation of transcription is associated with class-specific changes to the chromatin structure such that expression occurs at the necessary point throughout productive infection. In addition, upon viral DNA synthesis, although some newly replicated genomes do become associated with histones and histone-associated proteins, it is likely that the presence of a high number of viral genomes that do not become associated with histones provides an environment from which packaging of viral DNA can occur.

7. Final Discussion

7.1 Introduction

It is becoming increasingly clear that the regulation of gene transcription of a number of viruses is mediated by the association of histones and histone-associated proteins with viral DNA. Studies have shown that the differentiation-dependent control of HCMV gene expression that occurs during HCMV latency and reactivation is regulated by the chromatin status of the MIEP which drives expression of the viral major IE proteins that are crucial for productive infection. In undifferentiated, non-permissive HCMV infected cells, the MIEP is associated with H3K9me2 or HP1 β , markers of transcriptional repression, whereas in differentiated, permissive infected cells the MIEP becomes associated instead with high levels of acetylated histones (Murphy *et al.*, 2002; Reeves *et al.*, 2005a; Reeves *et al.*, 2005b; Ioudinkova *et al.*, 2006). This regulation of viral gene expression by the state of viral chromatin is not restricted to HCMV. There is good evidence that regulation of latency and reactivation of a number of other herpesviruses, including EBV, KSHV and HSV-1, is mediated by the chromatin structure of the viral major lytic gene promoters.

However, the control of gene expression of different classes of viral genes throughout a productive, lytic infection of fully permissive cells with HSV-1 has also been shown to be mediated by chromatinisation and remodelling of promoters (Herrera & Triezenberg, 2004; Kent *et al.*, 2004). Similarly, in the case of MCMV, productive infection appears to be regulated by the chromatinisation of the viral MIEP as the HDAC inhibitor TSA is able to relieve histone-associated repression of the promoter during infection (Tang & Maul, 2003). Furthermore, in the case of HCMV, the impairment in growth of an IE72-deficient mutant HCMV can be rescued with the use of HDAC inhibitors (Nevels *et al.*, 2004b). This repression has been hypothesised to be mediated by chromatin remodelling enzymes present at ND10 where the viral genome is deposited upon infection. Consequently, the remit for this thesis was initially to determine whether chromatinisation of the HCMV MIEP also occurs during productive infection and, if so, which viral and cellular factors may play a role in this process.

7.2 The MIEP of HCMV is chromatinised throughout productive infection

It is now well established that the transcriptional regulation of gene expression in eukaryotic organisms is mediated by the modulation or remodelling of chromatin structure associated with promoters and coding sequences of genes. Studies have led to a consensus that particular modifications at certain positions along gene promoters and coding regions are in fact hallmarks for repression (H3K9me2 and HP1 β) or activation (acetylated histone-H4) of gene expression and that they can be used to determine the transcriptional activity of the gene. ChIP analyses of HCMV infected cells showed clearly that the viral MIEP is associated with many of the histone markers described above throughout productive infection. As early as 3 hours post-infection, semi-quantitative PCR analysis showed that histones were recruited to the MIEP. RTQ-PCR also established that, although the promoter was associated with histones at this time, the total association of chromatin was lower than that observed at 24 and 96 hours post-infection. This is likely due to continued recruitment of histones until a later IE timepoint of infection.

The association of markers of transcriptionally active or repressed chromatin observed throughout lytic infection was consistent with the known temporal regulation of IE gene transcription. For instance, association of acetylated histone-H4 with the MIEP was maximal by 24 hours post-infection, consistent with a recently published study (Park *et al.*, 2007b), and this was also concomitant with the association of H3K4me2 - a marker of transcriptional activation and elongation (Schneider *et al.*, 2004). At a late timepoint of infection (96 hours), the MIEP became predominantly associated with the histone-associated marker of repression, HP1 β . This, in turn, is consistent with the known IE86-mediated negative auto-regulation of the MIEP at later times of infection through interaction via the *crs* element of the promoter and the recruitment of HDACs (Reeves *et al.*, 2006). Interestingly, late in infection, a low level of acetylated histone-H4 was still associated with the MIEP, consistent with Reeves *et al.* (2006). By this timepoint, viral DNA synthesis would have begun and, thus, newly synthesised viral genomes would have been subject to chromatinisation. However, PFA treatment of cells demonstrated little change to the quantity of histone association with the MIEP at 96 hours post-infection. Hence, although chromatinisation of newly synthesised viral genomes may occur (see section 7.9 for further discussion), it does not appear to alter the relative association of modified histones. Indeed, it is possible that the residual association of acetylated histone-

H4 with the MIEP by this timepoint is consistent with the known transcription and expression of the minor IE86 gene products, also known as IE2-p55 and IE2-p38 (see Fig. 1.9), which have been shown essential for full productive infection at late times (Jenkins *et al.*, 1994; Stenberg *et al.*, 1989). Hence, although the MIEP must be negatively auto-regulated by late times of infection to inhibit expression of IE86, which at high level is toxic to the infected cell, nevertheless transcription may still be occurring at a low level.

7.3 The MIEP is associated with repressive chromatin immediately upon infection

Although viral IE genes are accepted to be expressed immediately upon infection (Meier & Stinski, 1996), ChIP analysis showed that at 3 hours post-infection the MIEP was predominantly associated with markers of transcriptional repression such as H3K9me2 and HP1 β . This association occurred post-infection and most likely after entry of the viral genome to the nucleus since purified infectious virions were not found associated with any histone protein. This is consistent with a recent examination of the HSV-1 infectious particle (Oh & Fraser, 2008). Thus, although the MIEP begins transcription within the first few hours of infection within a permissive cell, it must first overcome control dictated by a repressive chromatin structure that is presumably mediated by intrinsic cellular repressors.

7.4 Repression of the MIEP immediately upon infection is mediated by HDACs

The observation that an initial environment of transcriptional repression exists during HCMV infection is consistent with a number of studies using HDAC inhibitors. For instance, viral IE gene expression and virus replication is increased by TSA during MCMV infection (Tang & Maul, 2003). TSA can also complement an IE72 mutant HCMV (Nevels *et al.*, 2004b). Indeed, other HDAC inhibitors, such as valproic acid (VPA), were shown some time ago to stimulate HCMV gene expression (Kuntz-Simon & Obert, 1995; Michaelis *et al.*, 2004; Michaelis *et al.*, 2005). The work presented in this thesis also found a correlation of HDAC activity with repression of the viral MIEP immediately upon infection. TSA treatment of fibroblasts, permissive for HCMV infection, caused increases in gene expression of viral IE genes, consistent with a recent study (Saffert & Kalejta, 2006). TSA also caused an increase in subsequent expression of early and late genes and also augmented viral DNA synthesis and production of infectious progeny. However, TSA, a hydroxamic acid based inhibitor, is a global inhibitor of class I and II HDACs. Consequently, it is not known which specific class or individual HDACs act to repress the

MIEP at this infection timepoint. Use of valproic acid has already implicated HDAC1 as a possible repressor. However, other HDAC inhibitors are also available, such as sodium butyrate and HC toxin, which have different specificities to those of TSA and VPA. A more direct method to analyse the role of individual HDACs might be to employ siRNA technology specific for each enzyme. This type of analysis would also decrease the unspecific effects of using a global HDAC inhibitor which may confound the investigation.

7.5 Repression of the MIEP immediately upon infection is controlled by hDaxx

Although it is clear that HDACs have a role in the production of a repressive chromatin structure at the MIEP immediately upon HCMV infection, these types of chromatin remodelling enzymes are usually recruited to promoters or genes by other transcription factors. The HCMV genome, after entry to the nucleus, is thought to locate near to, or at, ND10 and these are known sites of such chromatin remodelling enzymes. Certain components of ND10 are also known to interact with HDACs and one component, hDaxx, was of interest as its over-expression in normally permissive U373 cells is known to result in a complete abrogation of HCMV IE gene expression and virus replication after infection and that this appeared due to increased association of the MIEP with HP1 β which could be overcome with TSA (Woodhall *et al.*, 2006). hDaxx has been demonstrated to interact with a number of different chromatin remodelling enzymes including HDACs (Hollenbach *et al.*, 2002). Therefore, it was likely that hDaxx mediated repression of the viral MIEP in hDaxx over-expressing cells via recruitment of HDACs.

Indeed, degradation of hDaxx within the first 12 hours of HCMV infection is driven by pp71 interaction upon infection (Saffert & Kalejta, 2006). This necessary directed proteasomal degradation of hDaxx would be consistent with it acting as a repressor of IE gene expression. Experimental hDaxx down-regulation has also been shown to aid the replication of pp71 mutant HCMVs (Cantrell & Bresnahan, 2005; Cantrell & Bresnahan, 2006; Preston & Nicholl, 2006). However, the complete degradation of hDaxx, as postulated by Saffert & Kalejta (2006), does not appear necessary for IE gene expression: only partial degradation, as shown in this thesis and other studies (Tavalai *et al.*, 2008), or indeed the inhibition of any hDaxx degradation during HCMV infection by MG132 (Kaspari *et al.*, 2008) still leads to good levels of IE gene expression. Therefore, although the presence of hDaxx at the MIEP causes repression of IE gene expression, it may simply

be the removal of this repressor from this location, and not degradation, which is sufficient for pp71-driven transactivation. Additionally, the transient transactivation of the MIEP by pp71 may also be sufficient for immediate expression of IE72, which would lead to full disruption of the intrinsic intracellular anti-viral ND10 environment and, thus, a full productive infection.

An interesting finding of Park *et al.* (2007b) is that the level of HDAC2 protein in HCMV infected cells decreases concomitantly with the known degradation profile of hDaxx protein. It is known that HDAC2 turnover is controlled by the ubiquitin-proteasome pathway (Kramer *et al.*, 2003) and is possible that HDAC2, when associated with hDaxx, may be directed for proteasomal degradation by pp71 immediately upon infection. However, this specific hypothesis requires investigation. More intriguingly, Park *et al.* (2007b) observed increased levels of HDAC2 protein at delayed-early timepoints. As IE86 was also shown to interact with HDAC2 by their study, it is likely that HDAC2 may be involved in the negative auto-regulation of the MIEP at these times of infection, in addition to HDAC1 and the HMTs G9a and Suvar (Reeves *et al.*, 2006).

Although both TSA treatment and down-regulation of hDaxx prior to HCMV infection resulted in increases in viral gene expression, viral DNA synthesis and production of infectious progeny, these two increases were not equivalent. It is possible that other components of ND10 might recruit chromatin remodelling enzymes that are able to repress the MIEP in addition to HDACs recruited by hDaxx. Indeed, PML has been shown to interact with HDACs (Wu *et al.*, 2001c) and an independent contribution of PML to HCMV gene expression has been suggested (Tavalai *et al.*, 2006; Tavalai *et al.*, 2008) which may in part be due to this recruitment. Furthermore, the PML-associated repressor of transcription (PAROT), which is able to associate with all HP1 isoforms and, interestingly, is inhibited from association with DNA by TSA, can be recruited to ND10 by the PML-IV isoform (Fleisher *et al.*, 2006). Moreover, the possibility that independent but convergent mediators of repression of herpesvirus gene expression has been suggested for PML and Sp100 during HSV-1 infection (Everett *et al.*, 2008). More direct evidence of the involvement of PML in chromatin-mediated MIEP repression could be gained through ChIP analysis for the protein at the MIEP.

As well as HDACs, hDaxx is also able to recruit further chromatin remodelling enzymes to ND10, most notably the SWI/SNF chromatin remodelling family member ATRX (Ishov *et al.*, 2004; Tang *et al.*, 2004). ATRX contains a putative ATPase/helicase domain homologous to that found in SNF2 family members and a plant homeodomain (PHD) zinc finger domain similar to that of the DNMT3 protein family (Picketts *et al.*, 1996; Gibbons *et al.*, 1997). Although its function remains enigmatic, it is likely that the ATRX protein can function as a chromatin modifying enzyme. As recruitment of ATRX to ND10 is dependent upon hDaxx, it is likely that the two could contribute towards a repressive chromatin state at associated DNA:histone complexes. Therefore, the growing number of viruses that hDaxx appears to be able to repress, at least at some point during their life cycle (see section 4.3), makes it clear that hDaxx has an important role as a mediator of chromatin remodelling during herpesvirus infections.

As yet, there is no evidence for a direct physical interaction of hDaxx with any promoters which it has been shown to repress. It has been suggested that hDaxx does not interact with DNA directly but is targeted indirectly to promoters by the association of other factors: for example, hDaxx has been found to interact with core histones via the cellular Dek protein (Hollenbach *et al.*, 2002). Similarly, a recent report has shown the association of Daxx with an endogenous promoter. In this study, mDaxx was immunoprecipitated and showed an interaction with the murine c-Met promoter, which it is known to repress (Michaelson & Leder, 2003; Morozov *et al.*, 2008). Hence, it might yet be possible to show interaction of hDaxx with the MIEP of HCMV, most likely within a complex of proteins, through ChIP analysis, although the availability of ChIP assured antibodies for hDaxx must first be improved.

7.6 hDaxx down-regulation is not associated with an induction of HCMV IE gene expression in *in vitro* models of latency

The ability of hDaxx to mediate repression of viral IE gene expression at the earliest times of infection by chromatin-mediated changes raised the question as to whether repression by hDaxx could also mediate the differentiation-dependent control of MIEP activity observed during latency and reactivation. However, down-regulation of endogenous hDaxx protein in normally non-permissive T2 and THP1 cells was not associated with any induction in viral IE gene expression. Furthermore, the level of endogenous hDaxx in these cells and

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isolated monocytes did not decrease upon differentiation. The protein levels of transcriptional repressors such as YY1 and ERF also do not change upon differentiation of cells to a permissive form (Bain *et al.*, 2003; Wright *et al.*, 2005) and it is interesting to note that knockdown of these repressors also does not result in permissiveness for IE gene expression. However, the level of the chromatin remodelling enzyme, HDAC3, known to repress the MIEP in T2 cells does indeed decrease (Murphy *et al.*, 2002). Therefore, the presence or level of hDaxx protein does not appear to be involved in the switch from a latent infection to reactivation either.

In complete contrast to the findings presented in this thesis, Saffert & Kalejta (2007) did find a correlation between down-regulation of hDaxx, specifically in the T2 and THP1 cell lines, and an induction of HCMV IE gene expression. However, as described in detail in section 6.3, these observations could be explained by augmentation of IE gene expression in a spontaneously differentiating sub-population of T2 or THP1 cells after hDaxx down-regulation, similar to that shown in permissive human fibroblasts in Chapter 4. A crucial question that needs to be addressed is whether the increase in T2 and THP1 cells expressing IE72 after hDaxx down-regulation actually occurred in differentiated cells.

It is also interesting that the increase in IE gene expression observed by Saffert & Kalejta (2007) after hDaxx down-regulation did not lead to full productive infection. Instead, replication appeared to stall after expression of certain early viral genes. This is also true when the major IE proteins IE72 and IE86 are ectopically expressed in the THP1 cell line (Yee *et al.*, 2007) and would appear to show that even if leaky IE gene transcription did occur within undifferentiated cells, the cells would not allow full virus replication, most likely due to the transcriptional milieu of these undifferentiated cells (Sinclair & Sissons, 2006). It is also important to note that the use of the HDAC inhibitor VPA used by Saffert & Kalejta (2007) can result in differentiation of a number of cells types (Gottlicher *et al.*, 2001). This has been shown not to be the case with use of TSA treatment of the T2 cell line (Murphy *et al.*, 2002).

A criticism of both Saffert & Kalejta's (2007) work and that presented in this thesis is that the undifferentiated T2 cells commonly used for investigation of HCMV latency and reactivation do not possess normal ND10 (Hsu & Everett, 2001): the level of PML protein

within these cells is lower than that observed in most other cell types and leads to the preferential association of hDaxx with centromeres. In fact, normal ND10 structure is actually restored by differentiation of these cells (Hsu & Everett, 2001), posing the question of whether this cell type is a fair *in vitro* model of HCMV latency. Additionally, the theory put forward by Saffert & Kalejta (2007) that the lack of proteasomal degradation of ND10-associated hDaxx, due to the inability of pp71 to enter the nucleus, leads to a latent HCMV infection is difficult to reconcile with the fact that ND10 are not present in these cells. Furthermore, during experimental latent infection of CD34⁺ cells it appears that ND10 may be actively dispersed by latent HCMV gene products (McGregor-Dallas & Sinclair, unpublished data), yet IE gene expression does not occur within these cells until they are differentiated to DCs. Hence, from this analysis, hDaxx appears to have little role in the maintenance of latency or the control of reactivation of HCMV.

7.7 Experimental differentiation of cells is associated with an induction of HCMV IE gene expression in an *in vitro* model of latency

Whilst the down-regulation of hDaxx was associated with no induction of MIEP activity, the effort to control this study led to the discovery that down-regulation of the master regulator of differentiation, Oct4, did indeed lead to an induction of IE gene expression equivalent to that seen in RA differentiated cells. Although at first it was thought that permissiveness in Oct4 knockdown cells may be due to relief from Oct4 driven repression of the MIEP, a hypothesis made more interesting by the recent finding that Oct4 can form repressive complexes with HDACs and PML in some ES cells (Liang *et al.*, 2008), it quickly became clear that down-regulation of this master regulator of differentiation actually led to differentiation of T2 cells. The reciprocal experiment in which Oct4 was re-expressed in differentiated cells did not lead to repression of HCMV infection. This fits with recent data showing that additional cellular factors must also be re-expressed to de-differentiate cells (see section 5.3). These observations and other published studies suggest levels of chromatin remodelling or modifying enzymes and not necessarily the proteins which recruit them to promoters are intrinsically linked with the ability of conditionally permissive cells to support HCMV gene expression. This is also consistent with the fact that down-regulation of HP1 β is not in itself sufficient to induce IE gene transcription from the viral MIEP, most likely since chromatin remodelling enzymes such as HDAC3 are still present in high levels to maintain the latent infection of HCMV.

7.8 Regulation of all classes of HCMV genes is controlled by chromatin remodelling of promoters

The expression of HSV-1 genes had been shown to involve regulation through chromatin remodelling of promoters throughout infection and work in this thesis analysing prototypical early and late genes, UL44 and pp28 respectively, also showed modulation of their chromatin structure in a temporal fashion which coincided with known levels of gene transcription: when UL44 and pp28 transcription was not expected, markers of transcriptional repression (H3K9me2 and HP1 β) were associated with promoters. In contrast, at times when the genes were expected to be actively transcribed, a marker of transcriptional activation (acetylated histone-H4) was associated with their promoters. Pleasingly, a further marker of transcriptional activity and elongation, H3K4me2, used to demonstrate chromatin modification at viral promoters during HSV-1 infection (Kent *et al.*, 2004) was also associated with these promoters at times of active transcription. Other novel markers for active transcription at promoters have recently been identified, including ubiquitinated histone-H2A, and these may provide a better ability to demonstrate truly active promoters due to the very high turnover of these markers (Minsky *et al.*, 2008).

Despite the general correlation between the association of markers of active or repressed transcription at the UL44 and pp28 promoters, it appeared that an association with HP1 β did not occur to any appreciable level even during their repression. Instead, H3K9me2 association was found predominantly at timepoints prior to the induction of UL44 and pp28 transcription. This observation is indicative of chromatin structure found within domains of cellular chromosomes in which chromatin appears to be structured as facultative heterochromatin (Trojer & Reinberg, 2007) and is consistent with a 'poised state' with respect to transcription such that transcription can be activated upon the attainment of a threshold level of activators, in this case IE72 and IE86. This is in contrast to the seemingly terminal negative auto-regulation of the MIEP by IE86 at late timepoints of infection which results in the high association of HP1 β (Reeves *et al.*, 2006). Interestingly, neither the UL44 or pp28 promoter appeared to be negatively regulated at late times of infection. Although this may not be surprising for the pp28 promoter, it is unexpected for the early UL44 promoter. However, it is worth noting that association of acetylated histone-H4 has also been observed at late times of infection in a recent study of the HCMV UL54 promoter (Park *et al.*, 2007b). One reason which may explain this

observation at the UL44 promoter is the possible activation of an upstream cryptic promoter at late times (Leach & Mocarski, 1989). However, such an element is unknown within the UL54 promoter. Furthermore, parallel studies on HSV-1 gene promoters have shown that, although histone acetylation decreases at late timepoints, recruitment of markers of transcriptional repression such as H3K9me3 does not occur to any appreciable level except at the ICP0 promoter (Kent *et al.*, 2004). Additional analysis of other early and late promoters may shed some light on this aspect of the regulation of HCMV gene expression. However, whether specific repression of early and late promoters is necessary at late times of infection remains unclear. In addition, it would be interesting to examine whether IE gene promoters other than the MIEP may be differentially regulated since the MIEP is known to provide atypically high transcriptional activity and has such a fundamental role in HCMV infection.

Although the regulation of HCMV gene transcription is clearly related to association of modified histones and histone-associated proteins throughout infection and the mechanism by which this regulates the MIEP is relatively well understood, the mechanism by which early and late promoters become activated is relatively unclear. IE86, an activator of early and late promoters, is known to interact with the histone acetyltransferase (HAT) P/CAF (Bryant *et al.*, 2000) which may represent one mechanism by which early and late promoters are activated. IE72 and IE86 are also known to interact with HDACs and it has been shown that the sequestration of HDACs by IE72 may be important for the activation of the UL44 promoter (Nevels *et al.*, 2004b). Therefore, the most likely explanation for the premature activation of the UL44 and pp28 promoters in the presence of TSA or hDaxx down-regulation is the elevated levels of IE proteins due to loss of repression at the MIEP. TSA or hDaxx down-regulation may also promote an environment conducive to UL44 and pp28 promoter activation alone as well. It is also interesting to note that the association of RNA pol II, which binds to promoters before mRNA transcription initiates with the ICP0 promoter of HSV-1 occurs in the absence of *de novo* viral protein synthesis. In contrast, recruitment of RNA pol II to early and late promoters is dependent upon the expression of the IE ICP4 protein (Sampath & DeLuca, 2008). Thus, it appears that the active recruitment of RNA pol II appears necessary at early and late herpesvirus promoters in contrast to IE promoters, although this is unconfirmed during HCMV infection.

A further question which has yet to be addressed during HCMV infection is whether the histone:DNA structure termed chromatin throughout this thesis has the classical structure of nucleosomes. Early work on the control of HCMV gene expression appeared to show the association of chromatin or nucleosomal-like structures with viral DNA during infection (Kierszenbaum & Huang, 1978; St Jeor *et al.*, 1982). However, a definitive demonstration of classical nucleosomes at viral promoters or at coding regions of the viral genome has yet to be shown. The work of Kent *et al.* (2004) on HSV-1 productive infection has shown classical nucleosomal laddering upon micrococcal nuclease digestion of viral chromatin. Therefore, this type of analysis could be carried out during HCMV infection to determine whether regions of the viral genome apparently associated with modified histones are in a classical nucleosome structure.

7.9 The proportion of viral genomes associated with histones decreases during the course of productive infection

The observation that total levels of chromatin associated with all promoters (MIE, UL44 and pp28) analysed by ChIP decreased at 96 hours post-infection was consistent with a 2-fold reduction in the proportion of viral genomes associated with histone-H3 by late times of infection. At the earliest times of infection investigated, around 20% of incoming viral genomes were associated with histones. Although this is a sizable proportion of the incoming viral genomes, 80% of incoming genomes appear not to be associated with histones. The reason for this is unclear. However, it is also not known how many of these genomes are actually capable of initiating transcription. It is well established that particle to PFU ratios of many herpesviruses are high, reflecting a large proportion of virus particles which are defective for replication. This may be consistent with the proportion of genomes that appear not to be associated with histones. To test this hypothesis, an analysis of active RNA pol II association (Morris *et al.*, 2005) with HCMV promoters could be carried out to ascertain the proportion of genomes transcriptionally active. Although this might not directly implicate a certain genome population with transcriptional activity, if the proportion of genomes associated with RNA pol II correlated with a similar proportion of histone-H3 associated genomes, it may be concluded that the histone-associated population included the definitively active genomes. However, this awaits confirmation.

7.10 Could repression of HCMV gene expression be mediated by other factors or mechanisms?

The control of HCMV gene expression is clearly regulated by histone association during latency, reactivation and productive infection. One area of epigenetic control not yet discussed is DNA methylation. The methylation of DNA at CpG sites has long been associated with silencing of gene expression (Gilbert *et al.*, 2007) and the genomes of some viruses, including HPV, are known to become differentially DNA methylated during infection (Badal *et al.*, 2004). Since histone modification is known to be affected by changes in DNA methylation (Gilbert *et al.*, 2007) and histone modification plays such an important role in the control of HCMV gene expression, it is possible that modification of viral DNA methylation could be involved in control of viral gene expression. Indeed, hDaxx has been shown to interact with such DNMTs (Michaelson *et al.*, 1999; Muromoto *et al.*, 2004). However, in a model of HCMV latency and reactivation using rat cells with integrated MIEPs, it has been shown that the MIEP is resistant to DNA methylation and this is consistent with a lower than average level of CpG sites along the promoter (Boom *et al.*, 1987; Honess *et al.*, 1989). In support of these findings, analysis of the HCMV MIEP during reactivation studies found little DNA methylation in latently infected cells (Hummel *et al.*, 2007). However, DNA methylation has not yet been completely discounted as a mechanism of control of HCMV gene expression. Such analyses can be difficult to carry out but if DNA methylation does have a role in control of HCMV gene expression it is likely that this may be observed during latency. Consequently, the CD34⁺ cells of seropositive donors could be assessed using methylation specific PCR (MSP) or bisulphite sequencing during *ex vivo* reactivation. This analysis could also be carried out on viral DNA during a full productive infection.

An emerging mechanism by which both cellular and viral gene expression is known to be controlled is through the production of micro RNAs (miRNAs). miRNAs can function to down-regulate gene expression in a similar fashion to small interfering RNAs (siRNAs) through the RNA interference (RNAi) pathway. To date, over 100 novel miRNAs have been found to be expressed by DNA viruses, with targets present in both viral and cellular genes (Grey *et al.*, 2008). Approximately 13 miRNAs have been identified in the HCMV genome (Grey *et al.*, 2005). However, intriguingly, one of the miRNAs identified, miR-UL122-1, in addition to targeting the cellular MHC class I related chain B (MICB) (Stern-

Ginossar *et al.*, 2007), has been shown to target the expression of IE72 during productive infection (Grey *et al.*, 2007). Therefore, expression of miR-UL112-1 or other miRNAs may be involved in the control of viral gene expression during lytic infection as well as during latency and reactivation.

Although a number of markers of transcriptionally active or repressed chromatin can now be widely used to assess the activity of cellular and viral promoters, many more factors are known to be important in transcriptional control. For instance, although the nucleosomal structure comprises core histones H2A, H2B, H3 and H4, certain variants of histone proteins are known to exist. More specifically, histone-H3 has at least four variants (H3.1, H3.2, H3.3 and CENP-A), with histone-H3.3 known to accumulate in actively transcribing regions (Hake & Allis, 2006; Loyola & Almouzni, 2007). Since the histone-H3.3 chaperone HIRA is a known component of ND10 (Zhang *et al.*, 2005), it is possible that viral genomes are preferentially associated with histone-H3.3 upon infection of permissive cells due to enzymatic recruitment onto nascent DNA. The association of histones such as H3.3 could, therefore, be examined by ChIP analysis across a timecourse of HCMV infection as to gauge the importance of these additional factors in control of HCMV gene expression.

Certain chromatin modifying enzymes, such as G9a and Suvar, are known to be important during HCMV infection for IE86-mediated repression of IE gene expression at late times of infection (Reeves *et al.*, 2006). However, the mechanism by which the association of histone-associated proteins, such as HP1 β , could be removed from viral promoters to, for instance, activate the MIEP, remains unknown. Increasing numbers of chromatin remodelling enzymes are being identified and this information has lead to two possible mechanisms by which HP1 β could be displaced from H3K9me3 residues: phosphorylation of HP1 β directly by DNA damage response associated enzymes such as casein kinase 2 (CK2), which causes chromodomain interactions to decrease (Ayoub *et al.*, 2008); and phosphorylation of H3S10, adjacent to H3K9me3, by enzymes such as Aurora B kinase (AurKB) which causes HP1 β displacement (Fischle *et al.*, 2005; Hirota *et al.*, 2005). It is likely that many more chromatin modifying and remodelling enzymes exist and remain to be identified. As such, the number of different mechanisms by which chromatin modification could influence viral gene expression is also likely to increase.

7.11 Concluding remarks

Work from several groups, as well as work described in this thesis, has now gone some way to identifying both cellular and viral factors that control transcription from HCMV promoters, including the MIEP (see Fig. 7.1), and the mechanisms by which this occurs. These include chromatin-mediated regulation during reactivation from latency and also full productive infection. It is likely that HCMV has co-evolved with humans to usurp cellular mechanisms of repression imposed upon it during infection. HCMV is able to lie latent within the myeloid cells of an individual after primary infection due to their non-permissive, undifferentiated state and subsequent transcriptional milieu. This latent infection then enables asymptomatic persistence of the virus for the lifetime of the host. Transmission of HCMV to new recipients is then dependent upon reactivation of the virus from latency, most likely upon differentiation of the latently infected cell. Full lytic infection can occur after the virus overcomes any intrinsic intracellular repressors, which it is likely to face upon primary infection also. Efficient replication is then dictated by temporal regulation of viral gene expression throughout productive infection before transmission can occur. The success of this virus lifecycle is clearly indicated by the proportion of the human population asymptotically and latently infected with HCMV today.

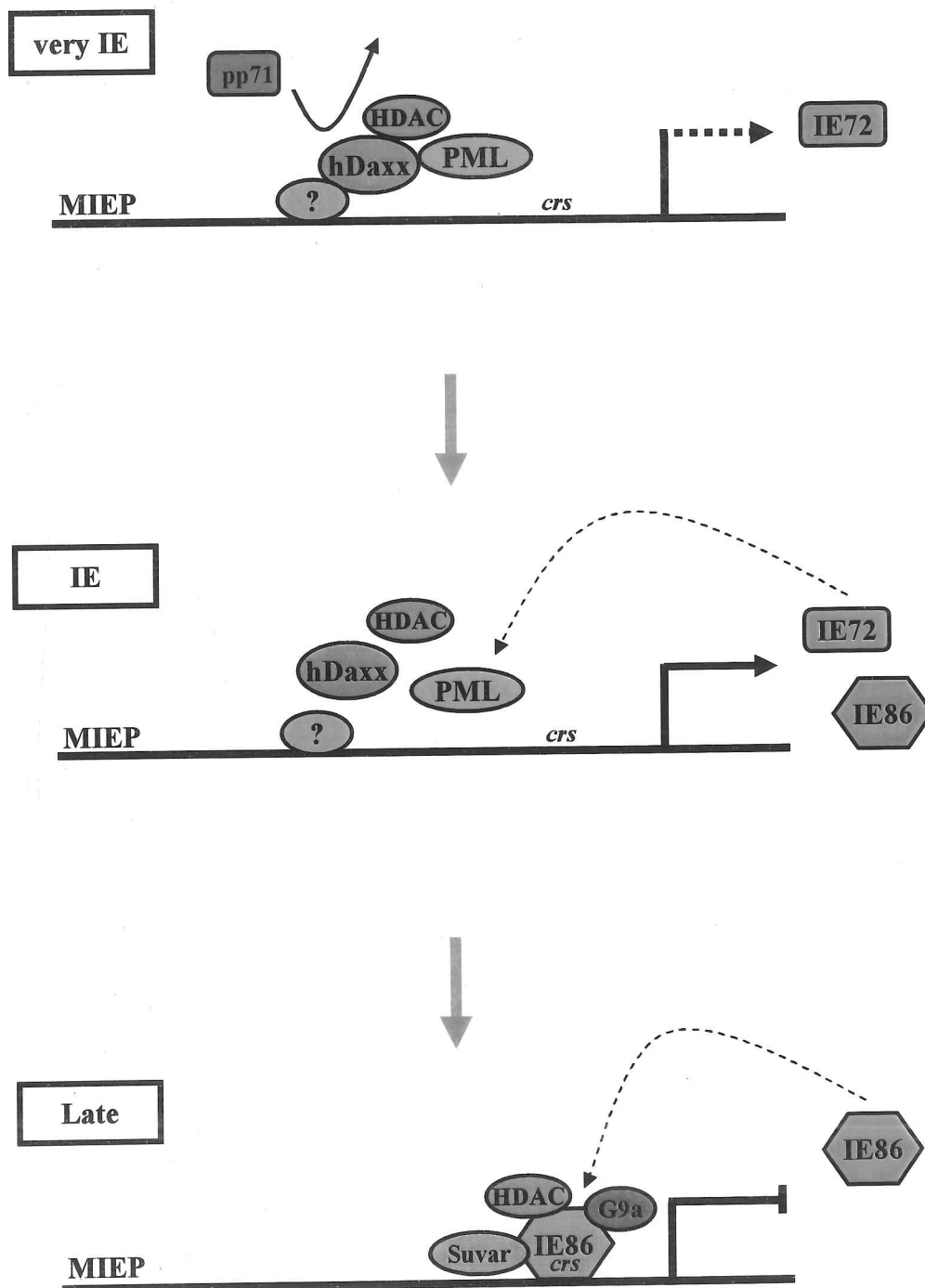


Figure 7.1 Current understanding of MIEP regulation throughout lytic infection. (very IE) Immediately upon HCMV infection of permissive cells the MIEP becomes associated with repressive chromatin structure through interaction with ND10 components. Incoming pp71 targets the MIEP via interaction with hDaxx and trans-activates MIEP activity, most likely by proteasomal degradation of hDaxx, which leads to transient major IE gene expression. (IE) The major IE gene product IE72 then disrupts the intrinsic anti-viral ND10 structure via interaction with PML, relieving repressive chromatin at the MIEP and further supporting IE gene expression. (Late) At a late timepoint, IE86 negatively auto-regulates the MIEP through binding to the *crs* element, resulting in chromatin-mediated repression of the MIEP.

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Reagents

ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
dCTP	2'-Deoxycytidine 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
dH₂O	Distilled water
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EMEM	Eagle's minimum essential medium
FAM	6-Carboxyfluorescein
FCS	Fetal calf serum
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GCV	Ganciclovir
HC	Hydrocortisone
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
LB broth	Luria-Bertani broth
LiCl	Lithium chloride
LPS	Lipopolysaccharide
MgCl₂	Magnesium chloride
MgSO₄	Magnesium sulphate
NaCl	Sodium chloride
NaClO₄	Sodium perchlorate
Na₂CO₃	Sodium carbonate
NaHCO₃	Sodium bicarbonate
NaH₂PO₄	Sodium dihydrophosphate
Na₂HPO₄	Disodium hydrophosphate
NP40	Nonidet P40
ONPG	Ortho-nitrophenyl-b-D-galactopyranoside
PFA	Phosphonoformic acid (Foscarnet)
PMA	Phorbol 12-myristate 13-acetate (TPA)
PMSF	Phenylmethylsulfonyl fluoride
RA	Retinoic acid
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride/sodium citrate solution

TBE	Tris/boric acid/EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TMR	6-Carboxy-tetramethyl-rhodamine
TPA	12- <i>O</i> -Tetradecanoylphorbol 13-acetate (PMA)
Tris	Trishydroxymethylaminomethane
TSA	Trichostatin A

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Short Communication

Knockdown of hDaxx in normally non-permissive undifferentiated cells does not permit human cytomegalovirus immediate-early gene expression

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The cellular protein human Daxx (hDaxx), a component of nuclear domain 10 structures, is known to mediate transcriptional repression of human cytomegalovirus immediate-early (IE) gene expression upon infection of permissive cell types, at least in part, by regulation of chromatin structure around the major IE promoter (MIEP). As it is now clear that differentiation-dependent regulation of the MIEP also plays a pivotal role in the control of latency and reactivation, we asked whether hDaxx-mediated repression is involved in differentiation-dependent MIEP regulation. We show that downregulation of hDaxx by using small interfering RNA technology in undifferentiated NT2D1 cells does not permit expression of viral IE genes, nor does it result in changes in chromatin structure around the MIEP. Viral IE gene expression is only observed upon cellular differentiation, suggesting little involvement of hDaxx in the regulation of the viral MIEP in undifferentiated cells.

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Human cytomegalovirus (HCMV) is the prototypic member of the subfamily *Betaherpesvirinae*. Primary infection of healthy, immunocompetent individuals, although usually asymptomatic, results in lifelong persistence of the virus (Ho, 1990). However, primary infection or reactivation in immunosuppressed individuals, such as transplant or human immunodeficiency virus/AIDS patients, can lead to serious disease (Drew, 1988; Rubin, 1990).

Like other herpesviruses, early in infection, the genome of HCMV becomes associated with nuclear structures known as nuclear domain 10 (ND10), which later become replication compartments (Ishov & Maul, 1996; Maul, 1998). ND10 are discrete, interchromosomal accumulations of specific cellular proteins, several of which are known transcriptional repressors (Negorev & Maul, 2001). Interestingly, the immediate-early (IE) proteins of several human herpesviruses are known to disrupt these structures (Everett, 2001). Indeed, the HCMV major IE protein IE72 has been found to be both necessary and sufficient for this disruption (Kelly *et al.*, 1995; Koriath *et al.*, 1996; Ahn & Hayward, 1997, 2000; Wilkinson *et al.*, 1998).

Other viral proteins also appear to target these nuclear structures. The HCMV tegument protein pp71 has also been found to associate with ND10 via interaction with hDaxx (Hofmann *et al.*, 2002; Ishov *et al.*, 2002). hDaxx is known to act as a promiscuous transcriptional repressor and probably controls gene expression via recruitment of histone deacetylases (HDACs) (Hollenbach *et al.*, 2002; Michaelson & Leder, 2003). Therefore, it has been

suggested that hDaxx may play some role in control of HCMV gene expression upon infection. Consistent with this, overexpression of hDaxx in permissive cells causes the abrogation of virus infection, whereas, conversely, downregulation of hDaxx in permissive cells increases gene expression and virus replication (Cantrell & Bresnahan, 2006; Preston & Nicholl, 2006; Saffert & Kalejta, 2006a; Woodhall *et al.*, 2006).

Other evidence also suggests that hDaxx plays an important role in the repression of infection. The recruitment of pp71 to ND10 has been found to cause the proteosomal degradation of hDaxx 3–12 h post-infection (Saffert & Kalejta, 2006a). In addition, pp71 knockout viruses or a pp71 mutant virus unable to bind to hDaxx have been found to result in low levels of IE gene expression upon infection. This is relieved if hDaxx expression is repressed prior to infection (Cantrell & Bresnahan, 2005, 2006; Preston & Nicholl, 2006). Furthermore, this repression of viral IE gene expression appears to be regulated through hDaxx-mediated changes to chromatin structure around the viral MIEP, as infection of fibroblasts in which hDaxx expression has been inhibited by small interfering RNA (siRNA) technology results in the association of the viral MIEP with transcriptionally active chromatin (Woodhall *et al.*, 2006). As expected, this regulation appears to involve HDACs, as HDAC inhibitors such as trichostatin-A (TSA) relieve this repression (Saffert & Kalejta, 2006a; Woodhall *et al.*, 2006).

hDaxx, therefore, plays an important regulatory role in the control of HCMV IE gene expression from the MIEP

during productive infection in permissive cells. However, chromatin structure also appears to play an important role in the control of viral IE gene expression in non-permissive cell types and in the differentiation-dependent control of latency and reactivation of HCMV (Sinclair & Sissons, 2006). Firstly, experimental infection of CD34⁺ cells and monocytes results in the MIEP becoming associated with markers of transcriptional repression (Reeves *et al.*, 2005a), namely heterochromatin protein-1 (HP1) (Bannister *et al.*, 2001), and is consistent with a lack of IE gene expression in these cells. In contrast, infection of monocyte-derived macrophage or mature dendritic cells (DCs) leads to an accumulation of acetylated histones around the MIEP, a marker of transcriptional activation (Strahl & Allis, 2000), and an increase in IE gene expression (Murphy *et al.*, 2002; Reeves *et al.*, 2005a). This regulation of gene expression from the MIEP also appears to take place in cells from naturally infected seropositive donors (Reeves *et al.*, 2005b). However, it is unknown whether hDaxx has any role in the differentiation-dependent regulation of IE gene expression during virus latency or reactivation.

A number of model cell systems exist that recapitulate the differentiation-dependent regulation of viral IE gene expression observed during latency and reactivation of HCMV. The embryonal carcinoma cell system NT2D1 (T2 cells), which are non-permissive for viral infection due to a block in major IE gene expression, can be differentiated with retinoic acid (RA) to a fully permissive phenotype, T2RA cells (Andrews *et al.*, 1984; Gonczol *et al.*, 1984; Lubon *et al.*, 1989). Differentiation leads to changes in the chromatin structure around the viral MIEP in HCMV-infected cells identical to those observed upon reactivation of endogenous latent virus from CD34⁺ stem cells differentiated to DCs (Murphy *et al.*, 2002; Reeves *et al.*, 2005b). Indeed, treatment of normally non-permissive T2 cells with TSA has also been found to cause permissiveness of these cells for productive infection, entirely consistent with the involvement of HDACs in the control of MIEP activity (Meier, 2001; Murphy *et al.*, 2002). Although T2 cells have been found to have abnormal ND10, with lower levels of PML protein (Hsu & Everett, 2001), these cells have been used by a number of laboratories to mimic changes in cellular function associated with latency and reactivation of HCMV. Therefore, we have sought to address whether knockdown of hDaxx in T2 cells has any effect on expression of viral IE genes during HCMV infection.

Firstly, we transiently downregulated cellular hDaxx expression in T2 cells through the use of siRNA technology described previously (Michaelson & Leder, 2003), supplemented with simultaneous transfection via Lipofectamine 2000 (Invitrogen) of a second hDaxx-specific siRNA (Dharmacon). Knockdown of hDaxx was confirmed 72 h post-transfection both by analysis of RNA levels by RT-PCR (Fig. 1a), using sense primer 5'-GACGGACATTT-CCTCTTCCA-3' and antisense primer 5'-TCTCATGCAC-TGACCTTTGC-3', and by analysis of protein levels by

Western blot (Fig. 1b), using an hDaxx-specific antibody (D7810; Sigma) as described previously (Woodhall *et al.*, 2006). hDaxx expression was reduced substantially in sihDaxx-transfected cells (Fig. 1a, b, lane 3), confirmed by serial dilution of both scramble siRNA-transfected and sihDaxx-transfected T2 protein samples (Fig. 1c, lanes 1–4), whereas hDaxx levels remained unaffected in mock- or scramble siRNA-transfected cells (Fig. 1a and b, lanes 1 and 2). The knockdown of endogenous hDaxx had no effect on the differentiation state of T2 cells on the basis of expression of Oct4, a POU domain transcription factor used routinely as a marker for undifferentiated cells (Rosner *et al.*, 1990), as assessed by RT-PCR using sense primer 5'-GCATACTGTGGACCTCAGG-3' and antisense primer 5'-CCAAGGTGATCCTCTTCTG-3', and by Western blot using an Oct4-specific antibody (ab19857; Abcam) (Fig. 1a, b, lanes 1–3). As can be seen in Fig. 1, little or no induction of IE RNA (Fig. 1a, lane 3) or IE protein (Fig. 1b, lane 3) occurred in hDaxx knockdown cells 24 h post-infection with HCMV strain Toledo. In contrast, T2RA cells showed both an absence of Oct4 expression due to differentiation and the presence of both IE RNA (Fig. 1a, lane 4) and protein (Fig. 1b, lane 4) upon infection. However, as the Western blot analysis suggested that around 10% of hDaxx protein still remains in sihDaxx-transfected T2 cells (Fig. 1b, c), we wanted to ensure that this residual hDaxx was not sufficient to maintain hDaxx-mediated repression of transcription. To this end, we used a nuclear factor κ B (NF- κ B) luciferase construct (NF- κ B-luc; Stratagene) (0.2 μ g per sample) that is known to be repressed by hDaxx and whose repression can be relieved by siRNAs to hDaxx (Michaelson & Leder, 2003). All transfections included a β -actin promoter-driven *lacZ* plasmid (0.5 μ g per sample) to normalize transfection efficiency. Fig. 1(d) shows that, 24 h post-transfection, the activity of the NF- κ B-luc reporter in sihDaxx-transfected T2 cells was increased by 4.5-fold compared with scramble siRNA-transfected T2 cells. In contrast, an interferon-responsive luciferase construct that is not NF- κ B-dependent (ISRE-luc; Poole *et al.*, 2006) (0.5 μ g per sample) showed no increase in activity in sihDaxx-transfected cells (Fig. 1d). Thus, the residual level of hDaxx observed in our transient knockdown analysis is insufficient to maintain specific repression of a known hDaxx-repressible promoter.

As knockdown of hDaxx in T2 cells had no effect on IE gene expression, we hypothesized that the viral MIEP would still be associated with transcriptionally repressive chromatin in these cells. Therefore, we carried out chromatin immunoprecipitation (ChIP) analysis of scramble siRNA- or sihDaxx-transfected T2 cells and T2RA cells infected for 24 h with HCMV exactly as described previously (Reeves *et al.*, 2005a). DNA-protein complexes were incubated with either control (1:200 dilution; Sigma-Aldrich), anti-acetylated histone H4 (1:200 dilution; Upstate Biotech) or anti-HP1 (1:200 dilution; Serotec) antiserum. The acetylation status of histones associated with the viral MIEP was then analysed by real-time quantitative PCR: the MIEP was

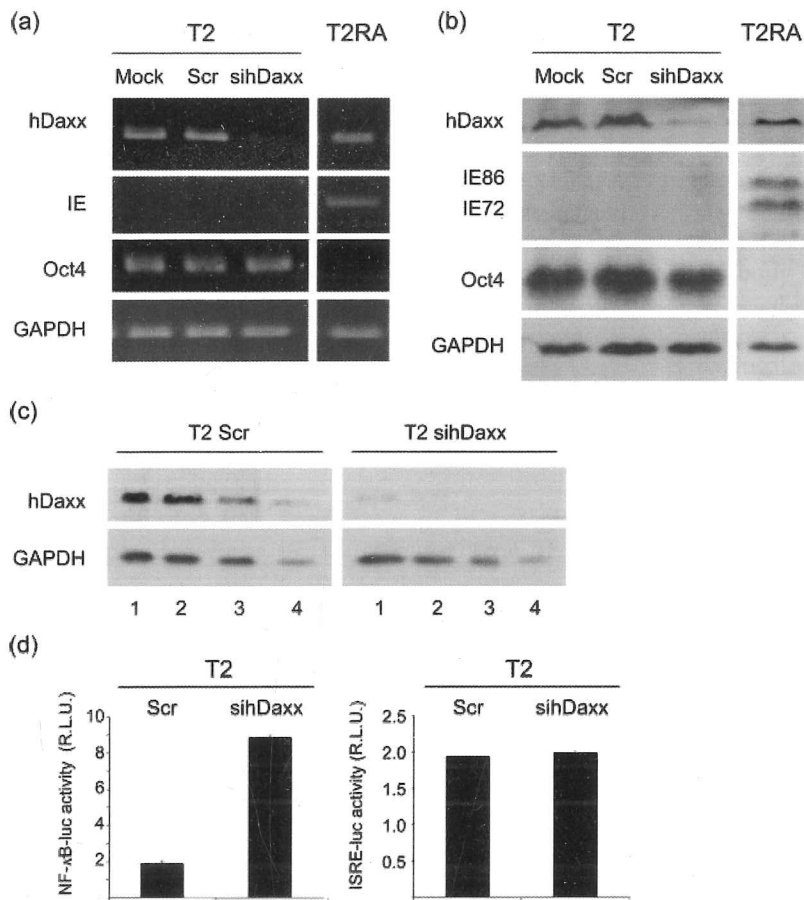


Fig. 1. Transient downregulation of hDaxx in T2 cells is not associated with the presence of viral IE gene expression upon HCMV infection. (a) RT-PCR analysis of total RNA isolated from mock-transfected, scramble siRNA-transfected (Scr) and sihDaxx-transfected infected T2 cells or infected, differentiated T2 (T2RA) cells for hDaxx, IE72, Oct4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels. (b) Western blot analysis of hDaxx, IE72/86, Oct4 and GAPDH expression in mock-transfected, scramble siRNA-transfected (Scr) and sihDaxx-transfected infected T2 cells or infected T2RA cells. (c) Western blot analysis of hDaxx and GAPDH expression in 20, 12, 6 and 2 μ l (lanes 1–4, respectively) scramble siRNA-transfected (Scr) and sihDaxx-transfected T2 cells. (d) Luciferase activity analysis of an NF- κ B luciferase (NF- κ B-luc) construct and an ISRE luciferase (ISRE-luc) construct in scramble siRNA-transfected (Scr) and sihDaxx-transfected T2 cells. Luciferase analysis was normalized by co-transfection with β -galactosidase. R.L.U., Relative light units. Error bars represent 1 sd of triplicate experiments.

amplified with sense primer 5'-CCAAGTCTCCACCCC-ATTGAC-3' and antisense primer 5'-GACATTTTGGAAGTCCCGTTG-3', complementary to positions -157 and -86 relative to the MIEP start site, and using a Taqman

probe (FAM-5'-TGGGAGTTTGT'TTTGGCACCAAA-3'-TMR). The MIEP in scramble siRNA-transfected T2 cells showed little association with acetylated histones (Fig. 2a, lane 3), but was associated predominantly with HP1 (Fig. 2a,

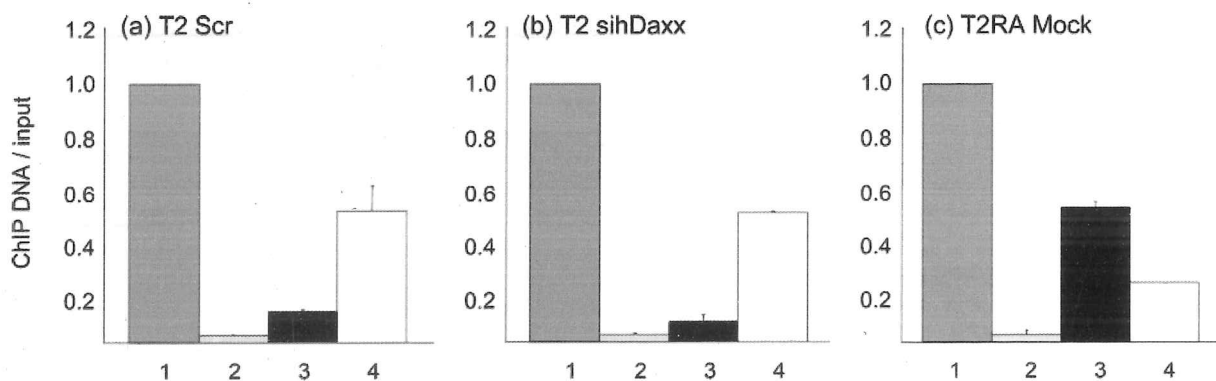


Fig. 2. Transient downregulation of hDaxx in T2 cells does not affect the chromatin structure of the viral MIEP and correlates with IE gene expression. ChIP assays were performed 24 h post-infection (m.o.i., 0.1) on (a) scramble siRNA-transfected T2 cells (Scr), (b) sihDaxx-transfected T2 cells and (c) mock-transfected T2RA cells with either control serum (lane 2), an anti-acetylated histone-H4 antibody (lane 3) or an anti-HP1 antibody (lane 4). Inputs are shown in lane 1. Isolated DNA was amplified by using an MIEP-specific real-time quantitative PCR. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. The data are representative of three independent experiments. Error bars represent 1 sd of triplicate PCRs.

lane 4), consistent with a lack of IE gene expression within these undifferentiated cells. T2 cells transfected with *sihDaxx* also showed the MIEP to be associated with low levels of acetylated histones (Fig. 2b, lane 3), but with high levels of HP1 (Fig. 2b, lane 4), again consistent with a lack of IE gene expression in these cells. In contrast, the viral MIEP in T2RA cells, permissive for IE gene expression, was associated predominantly with acetylated histones (Fig. 2c, lane 3) and little HP1 (Fig. 2c, lane 4), as we have shown previously (Murphy *et al.*, 2002). *hDaxx* knockdown in undifferentiated T2 cells, therefore, appears to have little effect on the chromatin status of the promoter, whereas equivalent levels of *hDaxx* knockdown in permissive fibroblasts have profound effects on the chromatin structure of the MIEP (Woodhall *et al.*, 2006).

We also confirmed that knockdown of *hDaxx* did not result in permissiveness of T2 cells for viral IE gene

expression by indirect immunofluorescence (Fig. 3a). T2 cells, transfected with either scramble or *hDaxx*-specific siRNAs for 72 h, and T2RA cells were infected with HCMV (at an m.o.i. of 0.5). After a further 24 h, cells were fixed (4% paraformaldehyde at room temperature for 10 min), permeabilized (70% ethanol at -20°C for 30 min) and stained for IE protein and Oct4 expression by using a mouse anti-IE72/IE86 antibody (clone E13; Argene) and an antibody specific for Oct4 (Abcam). Antibodies were detected by using an Alexa Fluor 594-labelled anti-mouse antibody (Invitrogen) and a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Sigma). Consistent with our Western blot analysis, neither *sihDaxx*- nor scramble siRNA-transfected cells showed evidence of viral IE gene expression. In contrast, T2RA cells showed high levels of permissiveness for IE gene expression (Fig. 3a), as expected. Differentiation of cells by RA was confirmed by co-staining for Oct4 (Fig. 3).

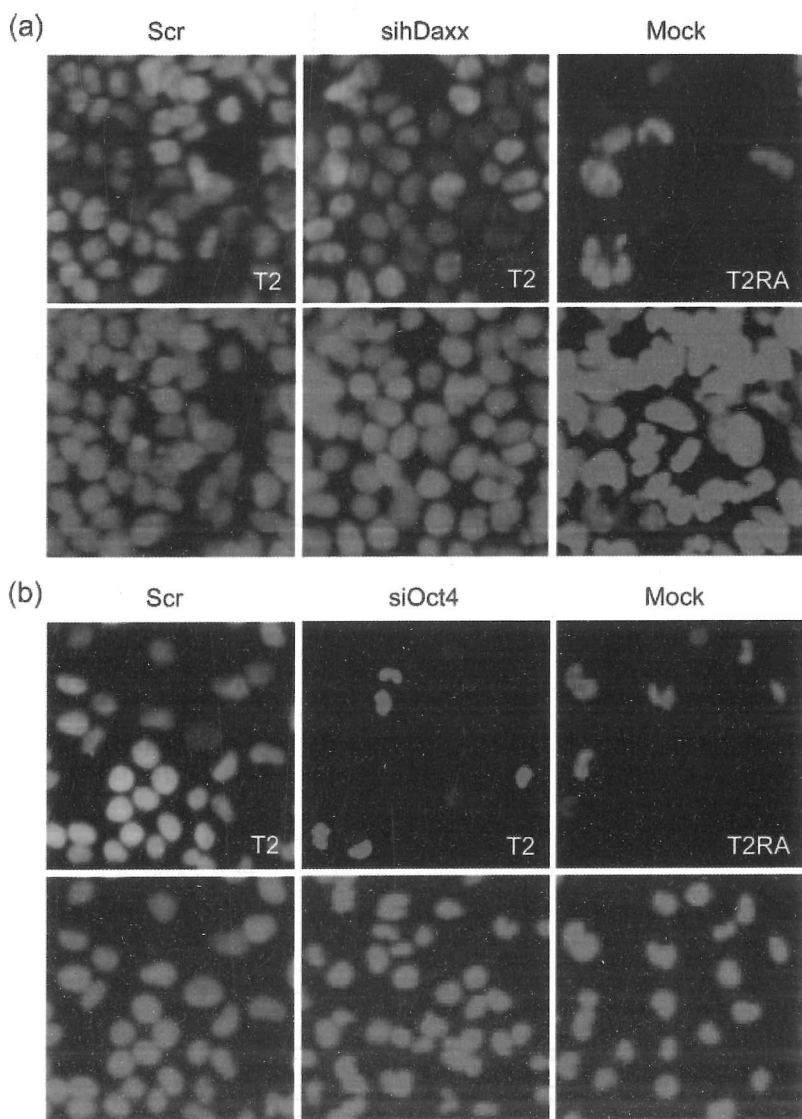


Fig. 3. Expression of HCMV IE protein is only associated with differentiated T2 cells. (a) Scramble siRNA-transfected (Scr) or *sihDaxx*-transfected T2 cells and T2RA cells were infected with HCMV (m.o.i., 0.5) and then stained for IE72/IE86 (red) and Oct4 (green) expression 24 h post-infection. (b) Scramble siRNA-transfected (Scr) or *siOct4*-transfected T2 cells and T2RA cells were infected with HCMV (m.o.i., 0.5) and then stained for IE72/IE86 (red) and Oct4 (green) expression 24 h post-infection. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.

HCMV can bind to and enter undifferentiated T2 cells, but cannot initiate IE gene expression (Meier, 2001), and this is probably due to the presence of high levels of transcriptional repressors and low levels of transcriptional activators of the MIEP (Sinclair & Sissons, 2006). Activators of the MIEP, such as NF- κ B, CREB and Sp1 (Hunninghake *et al.*, 1989; Sambucetti *et al.*, 1989; Lang *et al.*, 1992), are known to be at lower levels in undifferentiated cells than repressors such as YY1 and ERF, which also bind to sites in the MIEP (Liu *et al.*, 1994; Bain *et al.*, 2003; Wright *et al.*, 2005). In addition, some of these repressors are able to interact with specific co-repressors, such as HDACs and histone methyltransferases (Yang *et al.*, 1996; Wright *et al.*, 2005). Consequently, the transcriptional milieu of cells is likely to dictate whether infection with HCMV results in viral IE gene expression and productive infection. Indeed, specific knockdown of Oct4, a master regulator of differentiation, in T2 cells resulted in differentiation of these cells, as expected (Hay *et al.*, 2004; Matin *et al.*, 2004), and increased their permissiveness for HCMV IE gene expression (Fig. 3b). This also confirms that our knock-down technology can indeed result in T2 cells becoming permissive for IE gene expression.

In conclusion, although hDaxx has been implicated in repression of IE gene expression from the MIEP in permissive cell types, our results show that hDaxx does not control differentiation-dependent regulation of the viral MIEP in the conditionally permissive cell line NT2D1. IE gene expression and productive infection could only be seen in cells that were differentiated. We are aware that these results are in contrast to results of Saffert & Kalejta (2006b), who observed increased IE gene expression in undifferentiated cells after hDaxx knockdown; in collaboration, experiments are being carried out to address these discrepancies. However, it is also worth noting that levels of hDaxx protein do not differ significantly between non-permissive, undifferentiated cells and differentiated, permissive cells (data not shown), making it difficult to propose that levels of hDaxx protein alone control permissiveness in this model system. Consequently, our data support the argument that the permissiveness of cells for viral IE gene expression ultimately depends on the differentiation status of the infected cell.

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Human Daxx-mediated Repression of Human Cytomegalovirus Gene Expression Correlates with a Repressive Chromatin Structure around the Major Immediate Early Promoter*

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Upon herpesvirus infection, viral DNA becomes associated with nuclear structures known as nuclear domain 10 (ND10). The role of ND10 during herpesvirus infection has long been contentious; data arguing for a role for ND10 in repression of infection have been countered by other data showing little effect of ND10 on virus infection. Here we show that knockdown of human Daxx (hDaxx) expression, an important component of ND10, prior to infection with human cytomegalovirus resulted in increased levels of viral immediate early RNA and protein expression and that this correlated with an increased association of the major immediate early promoter with markers of transcriptionally active chromatin. Conversely, we also show that stable overexpression of hDaxx renders cells refractory to cytomegalovirus immediate early gene expression. Intriguingly, this hDaxx-mediated repression appears to be restricted to cells stably overexpressing hDaxx and is not recapitulated in transient transfection assays. Finally, hDaxx-mediated repression of cytomegalovirus major immediate early gene expression was overcome by infecting at higher virus titers, suggesting that an incoming viral structural protein or viral DNA is responsible for overcoming the repression of viral gene expression in hDaxx superexpressing cells. These data suggest that hDaxx in ND10 functions at the site of cytomegalovirus genome deposition to repress transcription of incoming viral genomes and that this repression is mediated by a direct and immediate effect of hDaxx on chromatin modification around the viral major immediate early promoter.

Human cytomegalovirus (HCMV)³ is an extremely widespread, opportunistic pathogen of considerable clinical significance.

Primary infection of healthy, immunocompetent individuals with HCMV is normally asymptomatic. However, as is the case with all herpesviruses, after the initial control of primary infection by the immune system, HCMV establishes lifelong latency in the host. During latency and throughout the lifetime of the infected individual, the herpesviruses can reactivate. Although generally asymptomatic, such reactivation can result in severe disease. As such, the disease burden of herpesviruses can be life-long and particularly severe when associated with immunosuppression, such as in transplant patients and in human immunodeficiency virus-infected patients who have developed immunodeficiency (1, 2).

After infection with herpes simplex virus-1, the viral genomes appear to co-localize with cellular nuclear structures known as nuclear domain 10 (ND10) and give rise to replication centers in close proximity to these sites (3, 4). ND10 are discrete interchromosomal accumulations of a number of proteins, many of which are transcriptional repressors. Intriguingly, many of the human herpesviruses encode an immediate early protein, which functions to destroy or redistribute components of ND10 (5–17), and a number of studies have attempted to address the role of ND10 during infection. One of the first observations was that ICP0 (intracellular protein 0) of herpes simplex virus-1 was sufficient for the disruption of ND10 (14) and that disruption occurred via proteosomal degradation of the promyelocytic leukemia (PML) protein, an essential structural component of ND10 (15, 18, 19).

Parallel studies with HCMV have identified that an immediate early protein, IE72, is similarly necessary and sufficient for the disruption of ND10 (5, 6, 11, 12, 16), although this disruption is mediated through an unknown mechanism and does not occur through ubiquitination and degradation via the proteasome (5, 20). In HCMV-infected cells, despite its relocation, PML has not been shown to be altered biochemically. Experiments that analyzed HCMV infection of a fully permissive astrocytoma cell line engineered to stably express PML (21), showed that disruption of ND10 by virus was delayed by up to 6 h compared with controls. Concomitant with this, viral DNA replication compartment formation was delayed, and the expression of early and late viral proteins were suppressed.

interfering human Daxx; simDaxx, small interfering mouse Daxx; EMEM, Eagle's minimal essential medium; H3-K9, histone H3 dimethylated at lysine residue 9; TRITC, tetramethylrhodamine isothiocyanate.

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³ The abbreviations used are: HCMV, human cytomegalovirus; ND10, nuclear domain 10; MIEP, major immediate early promoter; IE, immediate early; IE72 and IE86, immediate early protein 72 and 86, respectively; TSA, trichostatin A; MOI, multiplicity of infection; ChIP, chromatin immunoprecipitation assay; RFP, red fluorescent protein; pp71, phosphoprotein 71; PML, promyelocytic leukemia; siRNA, small interfering RNA; hDaxx, human Daxx; siDaxx, small interfering Daxx; mDaxx, mouse Daxx; sihDaxx, small

Overall, these data supported the suggestion that ND10 represents a nuclear environment which is unsupportive of viral gene transcription and that may function in an antiviral capacity. Consistent with this, a recent report has shown that the targeted knockdown of PML expression (and thus ND10 integrity) prior to viral infection results in the release of increased titers of progeny virus following productive infection (22).

The interaction of HCMV proteins with ND10 does not appear to be exclusive to IE72. The viral tegument protein and transactivator, pp71, accumulates at ND10 in a process mediated by another major ND10 component, the hDaxx protein (23–25). This results in a transient pp71-mediated degradation of hDaxx between 3 and 12 h of infection, arguing for a role of hDaxx in repression of infection (26). Consistent with this, hDaxx can function as a promiscuous transcriptional repressor, possibly via the recruitment of histone deacetylases to transcriptionally active chromatin (27), resulting in a closed chromatin structure and the repression of transcription (28–30).

It is becoming increasingly clear that herpesvirus gene expression may also be regulated by chromatin structure (31, 32). For example, the regulation of the major immediate early promoter (MIEP) is likely to involve higher order chromatin structure (32, 33). In experimentally infected monocytes and CD34⁺ cells, the MIEP is associated with HP-1 (heterochromatin protein 1), a classical marker of transcriptionally repressed chromatin (34), which is consistent with a lack of IE gene expression in these cells. In contrast, following infection of macrophages or mature dendritic cells, the MIEP is associated with acetylated histones, a marker of transcriptional activation (29, 30), and this correlates with detectable IE gene expression in these cells (32). Furthermore, this regulation of the MIEP by chromatin structure is also likely to be important during HCMV latency and reactivation in cells isolated from naturally infected seropositive individuals (35).

Although chromatin structure of the viral MIEP is clearly associated with latency and reactivation of viral IE gene expression, there is increasing evidence that chromatinization of the MIEP may also play a role during productive infection of fully permissive cell types. Although the virus and its major IE promoter exhibit only limited sequence homology with HCMV, in mouse cytomegalovirus there is evidence that chromatinization of the viral genome accompanies productive infection (36). The addition of the general histone deacetylase inhibitor trichostatin A (TSA) to permissive mouse embryonic fibroblast cells increased both the levels of murine IE1 gene expression and the titer of the virus recovered from these cells. Since one of the earliest events postinfection is the deposition of viral genomes at ND10 bodies (3, 37) and both the hDaxx and PML proteins interact with chromatin-remodeling enzyme activity (27, 38, 39), increased mouse cytomegalovirus IE gene expression induced by TSA upon deposition of the viral genomes to ND10 bodies may, at least in part, be due to the inhibition of histone deacetylases localized to ND10 bodies (36). Furthermore, we have observed that the MIEP of HCMV is also dynamically regulated by changes in post-translational modification of histones during productive infection of human fibroblast cells (40).

In this study, we have investigated the roles of hDaxx expression in the context of infection with HCMV. By the use of small

interfering RNA (siRNA), we show that knockdown of hDaxx expression in fully permissive cell lines prior to infection resulted in increased levels of IE RNA and protein expression in the siDaxx-treated cells and that this correlated with an increased association of the MIEP with markers of transcriptionally active chromatin. Conversely, we also show that stable overexpression of hDaxx or a red fluorescent protein-tagged hDaxx (RFP-hDaxx) renders cells refractory to HCMV immediate early gene expression. Intriguingly, this Daxx-mediated repression appears to be restricted to cells stably overexpressing hDaxx and is not recapitulated in transient transfection assays. Finally, hDaxx-mediated repression of HCMV major IE gene expression in cells stably overexpressing hDaxx was overcome by infecting at higher MOIs or by preincubating the cells with the histone deacetylase inhibitor TSA, suggesting that an incoming viral structural protein or viral DNA can overcome this hDaxx-mediated repression and that the observed Daxx-mediated repression of IE gene expression requires the recruitment of chromatin-modifying enzymes to the MIEP.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Human foreskin fibroblasts and human malignant glioma cells (U373 MG), were grown in Eagle's minimal essential medium (EMEM) (Invitrogen), supplemented with 10% (v/v) fetal calf serum with added penicillin/streptomycin (EMEM-10) at 37 °C in a humidified 5% CO₂ atmosphere. The low passage strain Toledo or AD169 was used for all HCMV infections. For infection with HCMV, cells were infected at room temperature for 1 h, with rocking, before the virus-containing medium was aspirated and replaced with fresh EMEM-10.

Plasmids and Transfections—pCDNA3hDaxx contains a full-length cDNA of hDaxx in the pCDNA3 expression vector. pDsRed (Clontech) encodes a novel RFP under the control of the minimal HCMV MIEP. pDsRed-hDaxx (a kind gift of Richard Caswell, Cardiff University) consists of the full-length hDaxx cDNA, minus the stop codon, cloned into EcoRI/BamHI-digested sites in pDsRed1-N1. To create cell populations transiently or stably expressing plasmid constructs, 25-cm² flasks were seeded with $\sim 1 \times 10^6$ cells 24 h prior to transfection. 2 h prior to transfection, cells were washed once with phosphate-buffered saline and overlaid with 4 ml of fresh EMEM-10. Each flask was transfected with 20 μ g of plasmid DNA using the CaCl₂ method. To select for stable transfectants, 24 h later, the EMEM-10 was replaced with fresh EMEM-10 containing 1 mg/ml G418 (Invitrogen), to select for cells expressing the Geneticin resistance cassette.

Immunofluorescent Microscopy—For immunofluorescence, 5×10^4 U373 cells/well were seeded on 8-well slides (Nunc) and infected at an MOI of 0.5–1, as described above. To detect pp65 protein at 8 h post-infection, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then permeabilized in 70% EtOH (–20 °C) for 15 min. Following washing, a mouse monoclonal anti-pp65 antibody (described in Ref. 41 and a kind gift of Bodo Plachter, Universität Mainz, Germany) diluted 1:10 in phosphate-buffered saline containing 1% bovine serum albumin was added to cells for 1 h at room temperature. Cells were then washed twice in phosphate-buffered saline and stained with rabbit

Daxx-mediated Repression of HCMV Gene Expression

anti-mouse fluorescein isothiocyanate (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

For PML, hDaxx, and IE72/IE86 staining, cells were stained as described above with either a rabbit anti-hDaxx antiserum (1:100 dilution; Upstate Biotechnology, Charlottesville, VA) for 1 h, a mouse anti-IE72/IE86 antibody (1:100 dilution; clone E13, Argene, Varilhes, France) or a mouse anti-PML antibody (1:200 dilution; Santa Cruz Biotechnology). hDaxx expression was then detected by staining with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:30 dilution; Sigma) or donkey anti-rabbit Alexafluor 594 (1:100 dilution; Sigma) for 1 h. PML or IE72/IE86 expression was detected using a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antiserum for 1 h (1:30 dilution; Sigma). All nuclei were counterstained with Hoechst 33242 (Sigma).

Cells were examined by UV microscopy, using the appropriate narrow band filters, in a Nikon Eclipse TE300 inverted microscope (Nikon UK Ltd.). Images were captured using a Hamamatsu color chilled 3CCD camera (Hamamatsu City, Japan), linked to the microscope, and Image Pro 4.1 software (Media Cybernetics UK).

Where specified, a minimum of 10 representative fields of view were counted for total cell number and the corresponding number of cells that were positive for hDaxx, RFP-hDaxx, or IE antigen expression as well as co-expression of IE antigen and hDaxx or RFP-hDaxx.

Chromatin Immunoprecipitation (ChIP) Assay—Chromatin immunoprecipitations were carried out essentially as previously described (32, 35). Briefly, fibroblasts were fixed with 1% formaldehyde to cross-link the DNA-protein interactions and then lysed. DNA associated with histones was immunoprecipitated with rabbit control serum (Sigma), anti-acetyl histone H4 antiserum (ChIP grade; Upstate Biotechnology), or anti-dimethylated (K9) histone H3 antiserum (Upstate Biotechnology) at a 1:200 dilution overnight at +4 °C.

DNA from disrupted nucleosomes was precipitated and amplified by PCR using the following conditions. The MIEP was amplified with sense primer (5'-TGG GAC TTT CCT ACT TGG-3') and antisense primer (5'-CCA GGC GAT CTG ACG GTT-3') complementary to positions -272 and +13 relative to the MIEP start site. The cycle parameters for amplification by MIEP-PCR were 20 cycles at 94 °C (40 s), 50 °C (40 s), and 72 °C (90 s). PCR products were transferred to nitrocellulose and hybridized to an MIEP-specific ³²P (Amersham Biosciences)-radiolabeled probe. The probe fragment was generated by PCR of HCMV DNA using sense primer (5'-ATT ACC ATG GTG ATG CGG TT-3') and antisense primer (5'-GGC GGA GTT GTT ACG ACA T-3'). Band intensity was analyzed using Image J analysis software.

Transient Down-regulation of Human and Murine Daxx Expression—siRNAs, previously shown to down-regulate hDaxx and mDaxx expression (42), were used in primary fibroblasts. The hDaxx siRNA was supplemented with a second hDaxx-specific siRNA (Dharmacon, Lafayette, CO) to maximize knockdown in subsequent experiments. All siRNAs were resuspended as 20 mM stocks. siRNAs were transfected into primary fibroblasts (passages 16–19) using Lipofectamine-2000 according to the manufacturer's instructions (Invitrogen).

Scramble siRNA (Dharmacon, Lafayette, CO) was used as a negative control siRNA duplex in these studies. The knockdown of hDaxx expression-transfected fibroblasts was assessed by Western blot analysis of total cell extracts.

Western Blotting—Western blot analyses of hDaxx, mDaxx, or IE72/IE86 expression were performed on total protein extracts of 10⁵ fibroblasts, which, following SDS-PAGE electrophoresis and transfer to nitrocellulose, were incubated with either a rabbit anti-hDaxx antiserum (1:4000 dilution; Sigma), a rabbit anti-mDaxx antiserum (1:200 dilution; Santa Cruz Biotechnology), or mouse anti-IE72/IE86 antibody (1:1200 dilution; clone E13; Argene, Varilhes, France) for 1 h and then a secondary horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse antiserum (1:2000 dilution; Sigma) for 20 min. Horseradish peroxidase activity was detected using ECL (Amersham Biosciences) as described by the manufacturer. The hDaxx and mDaxx proteins are detected as a 120-kDa band by SDS-PAGE.

Reverse Transcription-PCR—RNA was isolated from fibroblasts using RNazol B (Biogenesis, Poole, UK) as described by the manufacturer. Reverse transcription of 10 µg of total RNA was achieved using an avian myeloblastosis reverse transcriptase kit (Promega, Madison, WI) as described by the manufacturer. Primers designed to the IE region of the HCMV genome (between 172,468 and 172,776 nucleotides) were used to amplify a 194-bp fragment from cDNA using sense primer (5'-CGT CCT TGA CAC GAT GGA GT-3') and antisense primer (5'-ATT CTT CGG CCA ACT CTG GA-3'). The cycle parameters for amplification of immediate early cDNA were 20 cycles at 94 °C (40 s), 55 °C (40 s), and 72 °C (90 s). Cellular housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified using sense primer (5'-GAG TCA ACG GAT TTG GTC GT-3') and antisense primer (5'-TTG ATT TTG GAG GGA TCT CG-3'). The cycle parameters for amplification by glyceraldehyde-3-phosphate dehydrogenase-PCR were 20 cycles at 94 °C (40 s), 60 °C (40 s), and 72 °C (90 s).

RESULTS

It has been shown that a number of enzymes involved in chromatin remodeling are localized to ND10 bodies, including histone deacetylases and histone methyltransferases (27, 38, 39). The activity of the MIEP is regulated by higher order chromatin structure in both cell lines (32, 43) and primary myeloid cells during productive infection (32, 33) and during natural latency (35). More recently, we have observed that the MIEP is also associated with specific forms of chromatin during productive infection of fibroblasts,⁴ and thus we wished to determine whether repression of IE gene expression could be due to hDaxx mediating a repressive chromatin structure around the viral MIEP.

To do this, we used siRNAs to specifically target the down-regulation of endogenous hDaxx protein expression in primary human fibroblasts. Western blot analysis of hDaxx protein in siRNA-treated cells (Fig. 1A) clearly shows good down-regulation of hDaxx protein expression in cells transfected with sihDaxx (Fig. 1, A (lane 3) and D (lane 4)) and not in untreated

⁴ I. J. Groves, M. B. Reeves, and J. Sinclair, unpublished data.

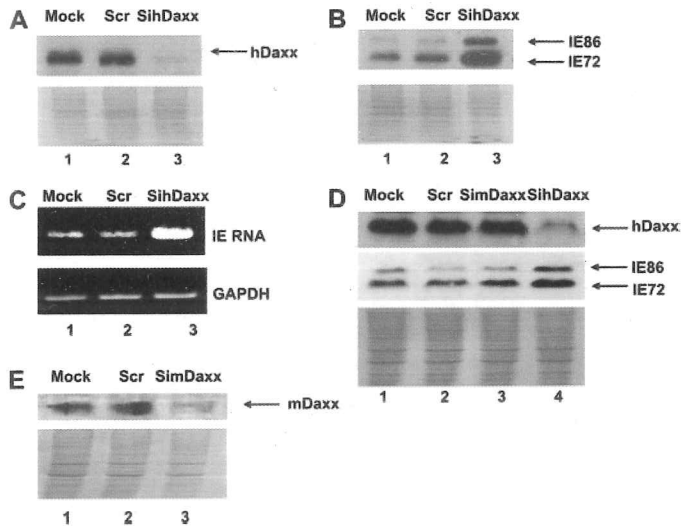


FIGURE 1. Transient down-regulation of Daxx correlates with changes in the activity of the viral MIEP in permissive cells. A, Western blot analysis of hDaxx expression in mock-transfected (lane 1), scramble-transfected (lane 2), and siDaxx-transfected (lane 3) primary human foreskin fibroblast cells. A Coomassie Blue-stained gel is shown as a protein loading control. B, Western blot analysis of mock-transfected (lane 1), scramble-transfected (lane 2), and siDaxx-transfected (lane 3) fibroblasts for IE72/IE86 protein expression 24 h postinfection at an MOI of 0.5. A Coomassie Blue-stained gel is shown as a protein loading control. C, reverse transcription-PCR analysis of total RNA isolated from mock-transfected (lane 1), scramble-transfected (lane 2), and siDaxx-transfected (lane 3) fibroblasts for IE72 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. D, Western blot analysis of hDaxx and IE72/IE86 expression in mock-transfected (lane 1), scramble-transfected (lane 2), siDaxx-transfected (lane 3), and siDaxx-transfected (lane 4) primary human foreskin fibroblast cells. A Coomassie Blue-stained gel is shown as a protein loading control. E, Western blot analysis of mDaxx expression in mock-transfected (lane 1), scramble-transfected (lane 2), and siDaxx-transfected (lane 3) primary mouse embryo fibroblast cells. A Coomassie Blue-stained gel is shown as a protein loading control.

(lane 1) or scramble siRNA-transfected fibroblasts (lane 2). This knock-down of hDaxx by specific siRNAs was maintained at similar levels up to 70 h post-transfection (data not shown). As additional controls for off-target effects of siRNA treatment, an siDaxx directed against the murine form of Daxx that resulted in the knockdown of murine Daxx in mouse embryonic fibroblasts (Fig. 1E, lane 3) had no effect on hDaxx expression in human fibroblasts (Fig. 1D, lane 3).

Having established that the transfection of siDaxx resulted in the substantial knock-down of hDaxx expression, cells transfected with siDaxx or scramble siRNA duplexes were subsequently infected with HCMV to determine the effect of hDaxx on HCMV infection. Western blot analysis for IE gene expression was performed on mock-, scramble siRNA-, siDaxx-, and siDaxx-transfected cells at 24 h postinfection at an MOI of 0.5. Infected fibroblasts transfected with hDaxx-specific siRNAs show increased levels of IE72 and IE86 gene expression (Fig. 1, B (lane 3) and D (lane 4)), compared with mock-transfected (lane 1), scramble siRNA-transfected (lane 2), or siDaxx-transfected (Fig. 1D, lane 3) controls. This increase in IE protein expression correlated with increased abundance of the IE72 RNA as detected by a concomitant reverse transcription-PCR analysis for IE expression (Fig. 1C). The data also shows that there are increased quantities of IE RNA present in cells at 24 h postinfection in siDaxx-transfected fibroblasts (Fig. 1C, lane 3) when

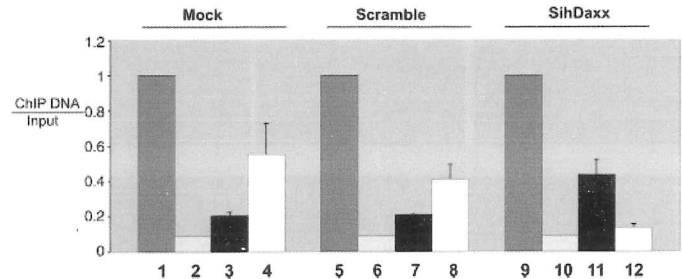


FIGURE 2. Transient down-regulation of hDaxx prior to infection results in a loss of repressive chromatin structure around the viral MIEP. ChIP assays were performed 3 h postinfection on mock, scramble, and siDaxx-transfected cells with an anti-acetylated histone H4 antibody (lanes 3, 7, and 11), an anti-dimethylated histone H3-K9 antibody (lanes 4, 8, and 12), or a rabbit isotype control (lanes 2, 6, and 10). Inputs are shown (lanes 1, 5, and 9). Isolated DNA was amplified using an MIEP-specific PCR and analyzed by Southern blot. For comparison, the immunoprecipitated DNA was quantified relative to the input DNA. Error bars, 1 S.D. of three independent experiments.

compared with either mock (Fig. 1C, lane 1) or scramble siRNA-transfected (Fig. 1C, lane 2) cells.

The levels of the IE72 and IE86 mRNAs detected in siDaxx-treated cells during HCMV infection is consistent with a model in which hDaxx acts to suppress transcription from the MIEP. Consequently, we asked whether knock-down of hDaxx in these cells correlated with an increase in the proportion of MIEPs associated with transcriptionally active chromatin. Chromatin was isolated from infected cells (MOI = 1) and analyzed by a ChIP assay specific for the viral MIEP (Fig. 2). At 3 h postinfection, the MIEP in untransfected control cells was associated with acetylated histones (Fig. 2, bar 3), as might be predicted for an immediate early promoter. We observed a similar pattern following the infection of scrambled siRNA-transfected fibroblasts (Fig. 2, bar 7). Interestingly, a substantial proportion of the viral MIEPs were also immunoprecipitated with histone H3 dimethylated at lysine residue 9 (H3-K9) (Fig. 2, bars 4 and 8). The methylation of H3-K9 is a highly characterized marker of transcriptionally silenced chromatin, and thus this observation suggests that a proportion of incoming viral genomes at this MOI are associated with repressive chromatin at 3 h postinfection. However, when we performed the same analysis in infected fibroblasts, which had been transfected with hDaxx-specific siRNAs, a different pattern of chromatinization was observed. Fig. 2 shows that a greater proportion of the MIEPs in siDaxx-treated cells were associated with an increased level of acetylated histones (Fig. 2, bar 11) and with much lower levels of dimethylated histones (Fig. 2, bar 12). Down-regulation of hDaxx prior to infection, therefore, appears to result in a discernible increase in the level of acetylation and concomitant decrease in the level of methylation of histones bound to the MIEP at 3 h postinfection.

hDaxx knock-down experiments strongly suggested that hDaxx was involved in repression of HCMV major IE gene expression as early as 3 h postinfection. We decided to determine, conversely, if overexpression of hDaxx could inhibit HCMV infection. To do this, we overexpressed hDaxx in U373 cells by transient transfection. Surprisingly, cells transiently overexpressing hDaxx were as permissive for HCMV (Fig. 3A), as control DsRed-transfected cells (data not shown) or the untransfected cells in the pcDNA3hDaxx-transfected popula-

Daxx-mediated Repression of HCMV Gene Expression

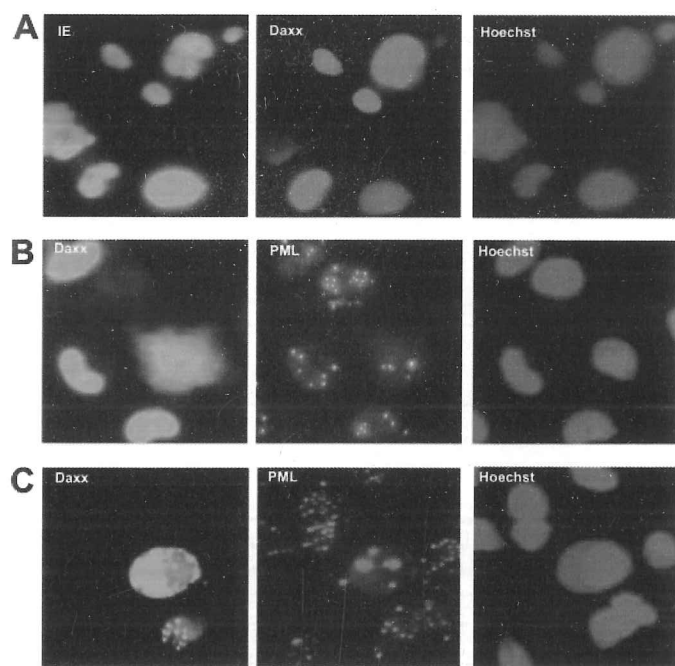


FIGURE 3. Transient overexpression of hDaxx has little effect on levels of superinfection with HCMV. A, U373 cell transiently transfected with pcDNA3Daxx were infected with HCMV at an MOI of 1 and then stained for hDaxx expression (*Daxx*) and viral IE gene expression (*IE*) 24 h postinfection. B and C, uninfected U373 cells transiently transfected with pcDNA3hDaxx were also stained for hDaxx (*Daxx*) and PML (*PML*) expression. In A–C, Hoechst 33342 staining was used to identify cell nuclei.

tion (*nonred cells* in Fig. 3A), as determined by IE gene expression. Consistent with observations from other workers, commercial anti-hDaxx antibodies are unable to detect endogenous hDaxx by indirect immunofluorescence but can detect hDaxx overexpressed by transfection. However, overexpressed hDaxx in transiently transfected cells was seldom detected associated with ND10 (see Fig. 3B), as might be expected. Co-localization of transiently expressed hDaxx with PML appeared only to occur in cells expressing relatively low levels of hDaxx (Fig. 3C). Such cells made up ~10% of the transiently transfected population (data not shown).

Since it is becoming increasingly clear that hDaxx function can be profoundly affected by its location, and in particular whether it is free or ND10-associated, we decided to also overexpress hDaxx by stable co-transfection (Fig. 4). Cautious of the differences we observed in hDaxx localization during transient transfection, we generated G418^r cells stably expressing hDaxx. To avoid nonrepresentative clones, a polyclonal population of G418^r hDaxx-expressing cells was used. In contrast to transiently expressed hDaxx, U373 cells stably transfected with hDaxx all exhibited punctate fluorescence, which co-localized with PML-stained ND10 (Fig. 4B). Interestingly, these stably transfected hDaxx cells were refractory to infection with HCMV (MOI = 1) as determined by viral IE gene expression (Fig. 4A). Direct quantification of the immunofluorescence shown in Fig. 4A revealed that stably superexpressing hDaxx cells exhibited an approximate 10-fold decrease in HCMV IE gene expression after superinfection (Fig. 4, A and C). We also quantified the level of overexpression of hDaxx in these cells by Western blot analysis (see Fig. 7A). On the basis that ~20% of

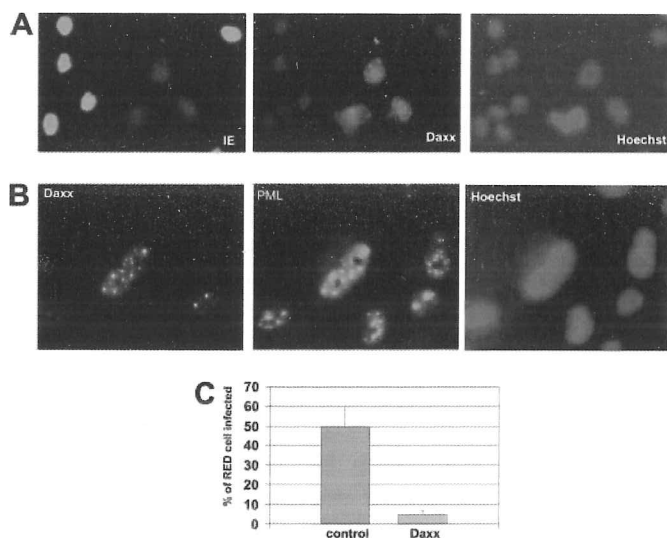


FIGURE 4. U373 cells constitutively overexpressing Daxx are refractory to infection with HCMV. A, U373 cells stably transfected with pcDNA3hDaxx were superinfected with HCMV at an MOI of 1. Cells were fixed and stained for hDaxx expression (*Daxx*) and viral IE gene expression (*IE*). B, these cells fixed and stained for hDaxx expression (*Daxx*) were also stained for PML expression (*PML*). A and B, Hoechst 33342 staining was used to identify cell nuclei. C, cells stably transfected with DsRed or pcDNA3hDaxx, superinfected with HCMV, were also co-stained for hDaxx and viral IE gene expression and analyzed by counting of representative fields; a minimum of 100 cells were counted from each of two independent experiments. The percentage of red cells (RFP- or TRITC-stained hDaxx-expressing cells) co-fluorescing green (IE-expressing cells) was quantified. Error bars, S.D.

cells discernibly overexpressed hDaxx by indirect immunofluorescence (Fig. 4A), Image J analysis of hDaxx band intensities of stably transfected cDNA3hDaxx *versus* stably transfected DsRed cells (Fig. 7A, *tracks 1* and *4*, respectively) suggest that hDaxx was overexpressed 3–4-fold in stably transfected cDNA3hDaxx cells.

In order to expedite analysis of co-expression of superexpressed hDaxx and viral IE gene expression, we also constructed a plasmid vector in which hDaxx was expressed as in frame with red fluorescent protein, generating RFP-tagged hDaxx (RFP-hDaxx). To do this, we stably transfected U373 cells with DsRed and DsRed-hDaxx (Fig. 5) and investigated the effects of RFP-hDaxx stable expression on infection with a HCMV. Once again, we generated G418^r nonclonal populations of cells stably expressing RFP and RFP-hDaxx to minimize the risk of selection of nonrepresentative cell clones. Stably, overexpressed RFP-hDaxx exhibited punctate nuclear expression (Fig. 5, A and B) characteristic of ND10, consistent with our observations in U373 cells stably transfected with untagged hDaxx. Interestingly, transiently transfected DsRed-hDaxx again showed that, in most cells, the RFP-hDaxx did not co-localize with PML except in cells expressing very low amounts of transfected RFP-hDaxx (data not shown), again suggesting that analyses in which hDaxx is transiently transfected into cells may be difficult to interpret. In contrast to our transient transfection data, we clearly observed that stably, overexpressed RFP-hDaxx localized to ND10 by immunofluorescent staining for endogenous PML protein (Fig. 5B). The punctate staining of both RFP-hDaxx and PML fully co-localized, confirming that in DsRed-hDaxx stably transfected cells RFP-hDaxx localized predominantly to ND10 bodies (Fig. 5B). In contrast, RFP

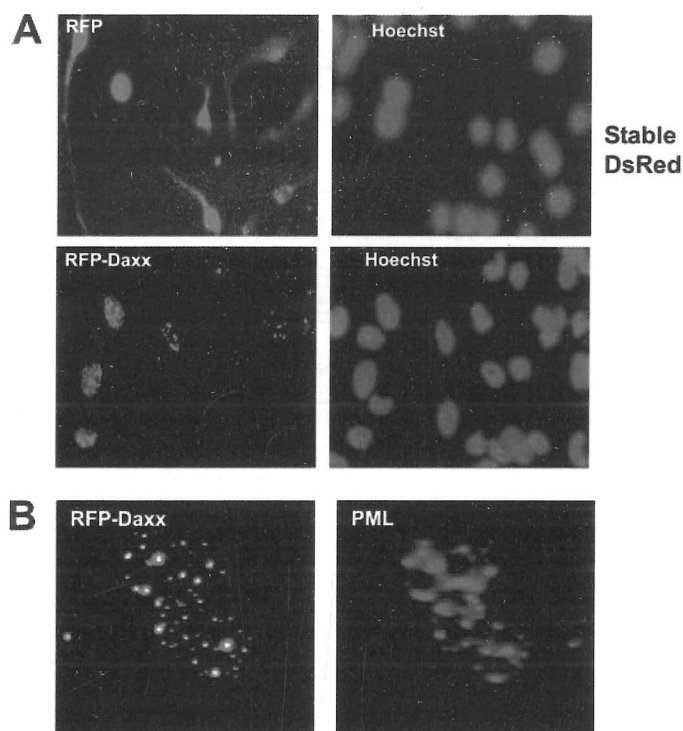


FIGURE 5. Cells stably expressing RFP-Daxx show co-localization of RFP-Daxx to bona fide ND10 bodies. A, U373 cells stably transfected with DsRed and expressing RFP (RFP) or stably transfected with DsRed-hDaxx and expressing RFP-hDaxx (RFP-Daxx) were analyzed by fluorescent microscopy. B, cells stably expressing RFP-hDaxx were also co-stained for PML (PML). Hoechst 33342 staining was used to identify cell nuclei.

expressed in DsRed-transfected cells showed diffuse cytoplasmic and nuclear expression (Fig. 5A), entirely different from RFP-hDaxx localization.

To test whether expression of other ND10 components was affected by RFP or RFP-hDaxx in stably transfected cells, Western blot analyses were performed on untransfected U373 cells or U373 cells transfected with DsRed or DsRed-hDaxx. Analysis of nuclear extracts showed that endogenous levels of hDaxx were equal in all of the three cell lines, as were levels of Sp100 and PML (data not shown). Based on a comparison of the number of cells in the stably transfected DsRed-hDaxx population overtly expressing RFP-hDaxx (~16%; see Fig. 5A) and Western blot analysis of endogenous hDaxx *versus* RFP-hDaxx (as was carried out for stably transfected cDNA3hDaxx cells, above), RFP-hDaxx was overexpressed ~2-fold with respect to endogenous Daxx in stably transfected DsRed-hDaxx cells (Fig. 7B).

We then investigated the effects of this RFP-hDaxx overexpression on HCMV infection. U373 DsRed- and U373 DsRed-hDaxx-transfected cells were infected with HCMV at an MOI of 1. At 18 h postinfection, cells were fixed and stained for IE gene expression and visualized by fluorescence microscopy for the co-expression of IE72, RFP, and RFP-hDaxx proteins. The U373 DsRed cells were fully permissive for HCMV IE gene expression (Fig. 6A, b). However, in U373 cells overexpressing RFP-hDaxx, no IE72 expression could be detected (Fig. 6A, e). This effect was specific to DsRed-hDaxx-transfected cells, since in the same field of view, cells that were not overexpressing RFP-hDaxx expressed detectable levels of IE72. Consistent with our observations with untagged hDaxx, these data suggested

that HCMV IE gene expression is precluded by high levels of RFP-hDaxx expression (Fig. 6A, e) but not RFP (Fig. 6A, b).

In order to rule out the possibility that U373 DsRed-hDaxx-transfected cells were refractory to virus binding and internalization, we also infected and analyzed them for expression of HCMV tegument protein, pp65. By indirect immunofluorescence, pp65 tegument protein could clearly be visualized within U373 RFP-Daxx-expressing cells (Fig. 6A, h), precluding the possibility that the repressive effects of RFP-Daxx overexpression on virus IE gene expression results from effects on virus binding or entry.

We also quantified the number of RFP- or RFP-hDaxx-expressing cells co-expressing viral IE72 in the polyclonal populations of U373 cells transfected with either DsRed or DsRed-hDaxx, respectively. Fig. 6B shows that cells that stably overexpressed RFP-hDaxx also showed a 7-fold decrease in their ability to support HCMV IE gene expression after superinfection. This repression was less pronounced in cells infected at higher MOIs, such that the 7-fold decrease in HCMV IE gene expression observed in hDaxx-RFP-expressing cells infected at an MOI of 1 was substantially reduced by infection at an MOI of 10 (Fig. 6C), suggesting that hDaxx-mediated repression of the MIEP in cells stably expressing RFP-hDaxx can be titrated away by increasing MOIs. Indeed, consistent with this, a Western blot analysis of hDaxx protein levels at 3 h postinfection of cDNA3hDaxx and DsRed stably transfected cells showed that HCMV-induced degradation of hDaxx protein required higher viral titers in cells stably transfected with cDNA3hDaxx (Fig. 7A). Consistent with published data (26), hDaxx degradation was induced by HCMV infection of control U373 cells stably transfected with DsRed at both 0.5 and 5 MOI (Fig. 7A, lanes 2 and 3) when compared with mock-infected cells (Fig. 7A, lane 1). In contrast, HCMV-induced hDaxx degradation in cells stably overexpressing hDaxx was only observed at higher MOIs (Fig. 7A, lane 3) and not lower MOIs (Fig. 7A, lane 2) when compared with mock-infected controls (Fig. 7A, lane 1). Similarly, in RFP-hDaxx-expressing cells, only high titers of virus were able to result in degradation of RFP-hDaxx or endogenous hDaxx in these cells (Fig. 7B).

Our ChIP analyses of the MIEP (Fig. 2) suggested that hDaxx-mediated repression of the IE gene expression could be associated with the known interaction of hDaxx with chromatin-modifying enzymes, since the knockdown of hDaxx resulted in a concomitant increase in the proportion of acetylated promoters following infection. Therefore, we reasoned that if hDaxx requires chromatin remodeling enzymes to repress the MIEP then inhibition of histone deacetylase activity could abrogate the ability of hDaxx to repress the MIEP.

First, a polyclonal population of RFP-hDaxx cells was infected at an MOI of 0.2, and the level of infection was calculated in the red and nonred populations. As expected, ~15% of nonred cells (*i.e.* not overexpressing hDaxx) expressed IE72 (Fig. 6D, bar 1). In contrast, we observed that 0.3% of RFP-hDaxx cells were expressing IE72 (Fig. 6D, bar 2), entirely consistent with data in Figs. 4 and 6. The same analysis was then performed on cells incubated with TSA prior to infection. Our analysis of the nonred cells showed that 60% of cells were infected and thus TSA itself induced a ~4-fold increase in the

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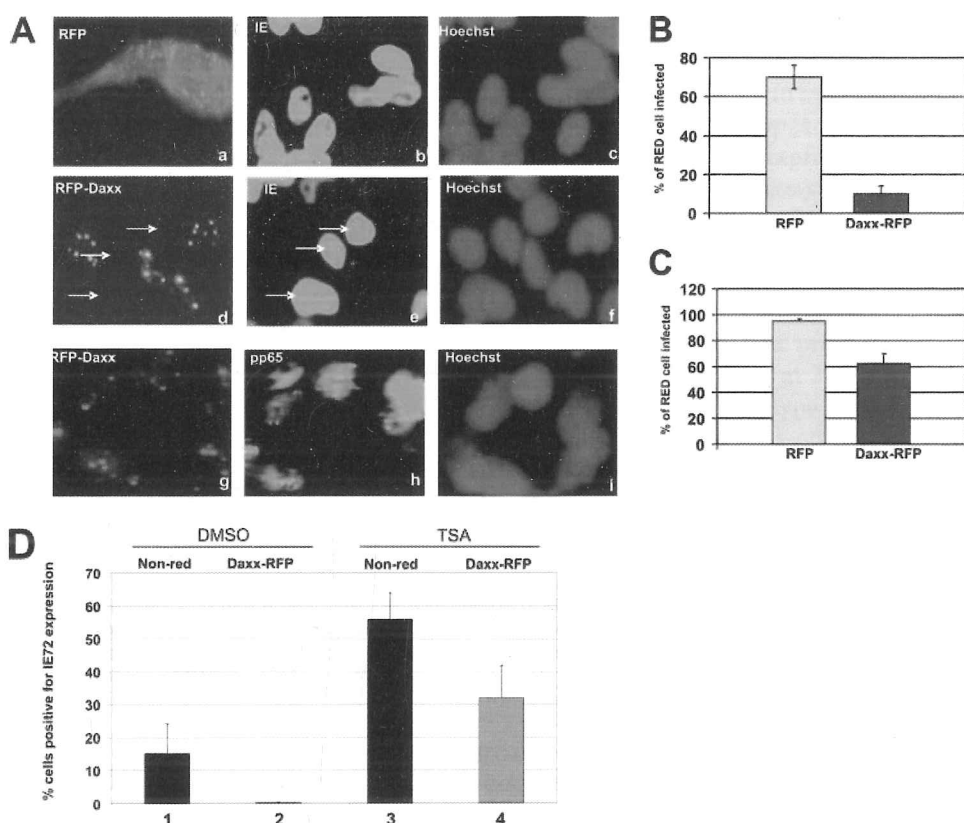


FIGURE 6. U373 cells constitutively overexpressing RFP-hDaxx are refractory to infection with HCMV. A, U373 stably transfected with DsRed (RFP) (a–c) or DsRed-hDaxx (RFP-hDaxx) (d–f and g–h) were infected with HCMV at an approximate MOI of 1. Cells were then stained with an anti-IE antibody (IE) (b–e). The arrows identify cells overexpressing RFP-hDaxx in the appropriate panels. Alternatively 6–8 h postinfection RFP-hDaxx expressing (g) cells were fixed and stained for pp65 expression (pp65) (h). Hoechst 33342 staining was used to identify cell nuclei. B, RFP- and RFP-hDaxx-expressing cells, infected with HCMV at an MOI of 1, were also stained for viral IE gene expression and analyzed by counting of representative fields; a minimum of 100 cells were counted from each of two independent experiments. The percentage of red cells (RFP- or RFP-hDaxx-expressing cells) co-fluorescing green (IE-expressing cells) was quantified. The error bars represent S.D. C, U373 stably transfected with DsRed-hDaxx cells were also superinfected with HCMV at an MOI of 10 and analyzed as described in B, above. D, a polyclonal population of RFP-hDaxx cells (20% expressing RFP-hDaxx) were incubated with control (lanes 1 and 2) or TSA (lanes 3 and 4) 18 h prior to infection. 24 h postinfection, the number of RFP-hDaxx (lanes 2 and 4) and non-RFP-hDaxx cells (lanes 1 and 3) expressing IE72 was quantified by fluorescent microscopy. Error bars, 1 S.D. DMSO, Me₂SO.

level of infection (Fig. 6D, bar 3), consistent with previous observations made during murine (36) and human⁴ CMV infection. Interestingly, however, 33% of RFP-hDaxx were also expressing IE72, representative of a 100-fold increase in the level of permissiveness of the RFP-hDaxx cells (Fig. 6D, bar 4). Although the addition of TSA did result in a 4-fold increase in infection in nonred cells, this was modest compared with the effect on RFP-hDaxx cells (100-fold increase). Thus, these data support the hypothesis that the observed hDaxx mediated repression of the MIEP is regulated, at least in part, by chromatin remodeling enzymes, since their inhibition with TSA counters the effects of hDaxx overexpression. Furthermore, these data confirm that the RFP-hDaxx cells retain the capacity to be productively infected and are not irreversibly compromised by RFP-hDaxx overexpression.

DISCUSSION

The hDaxx protein physically interacts with various proteins of the chromatin matrix, including histone deacetylase II, H2A, H2B, H3, and H4, and with the chromatin-associated protein

Dek (27, 38) based upon the phosphorylation status of hDaxx (27), and hDaxx is thought to recruit histone deacetylases to sites of active transcription by direct interaction with the transcription machinery. Histone tails are subsequently deacetylated. Histone deacetylation then results in a closed chromatin structure and the repression of transcription. Consistent with this, we observed that the HCMV MIEP was associated with dimethylated histones following the infection of synchronous cells. In hDaxx knock-down cells, histones were still associated with the MIEP upon infection; however, there was an overt difference in the pattern of chromatinization of the MIEP such that the MIEP was predominantly associated with acetylated histones. Consistent with this, analysis of the levels of viral IE gene expression also showed that hDaxx knock-down cells expressed considerably higher levels of viral major IE RNA and protein.

Conversely, overexpressed hDaxx could inhibit viral IE gene expression after virus infection. Either wild-type Daxx or RFP-Daxx when stably overexpressed in U373 cells was shown to localize predominantly to ND10 bodies. Both indirect immunofluorescence and Western blot analysis showed that these cells overexpressing hDaxx were sub-

stantially less able to support viral IE gene expression when infected at low multiplicities. This would be consistent with the proposed role of hDaxx as a promiscuous transcriptional repressor (38, 44–47), and when overexpressed, hDaxx would be present at high levels at ND10, a location in close proximity to herpesvirus genome deposition (3, 4). This hDaxx-mediated repression of viral IE gene expression was not due to an inability of cells to bind and internalize virus, since hDaxx superexpression did not affect uptake of HCMV tegument protein pp65. Furthermore, we could render these cells permissive for HCMV gene expression by infecting at higher MOIs or by incubating the cells with TSA, confirming that these cells are not just a subpopulation of cells that are irreversibly refractory to HCMV infection.

Intriguingly, we saw no such inhibition of viral IE gene expression after superinfection when hDaxx was transiently overexpressed in cells. We are not sure how the functionality of hDaxx differs between cells that are transiently expressing or stably overexpressing this protein. However, it has been suggested that hDaxx function can be profoundly affected by its

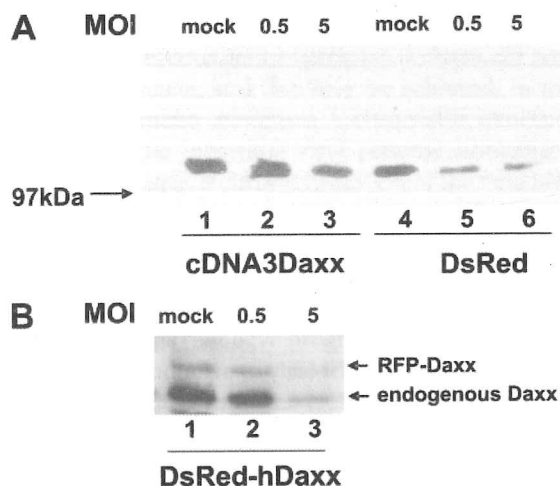


FIGURE 7. Western blot analysis of hDaxx protein expression in transfected cells. A, cDNA3hDaxx stably transfected U373 cells (tracks 1–3) or control DsRed stably transfected U373 cells (tracks 4–6) were left uninfected (mock) or infected at MOIs of 0.5 or 5 for 6 h and analyzed by Western blot for hDaxx expression. B, DsRed-hDaxx stably transfected U373 cells expressing RFP-hDaxx were left uninfected (mock) or infected at MOIs of 0.5 or 5 for 6 h and analyzed by Western blot for hDaxx expression. The arrows designate endogenous Daxx- and RFP-tagged Daxx.

location (48) and in particular whether it is free or ND10-associated. We note that transiently overexpressed hDaxx only resulted in association of the protein with ND10 structures infrequently, and it is possible that this may explain the activation of the MIEP by hDaxx observed using transient co-transfection assays (23). We are presently investigating the differences between transiently and stably overexpressed hDaxx in transfected cells but note that even cells expressing transiently transfected hDaxx at low levels, in which hDaxx does co-localize with ND10, do not show repression of viral IE gene expression (data not shown). Indeed, a recent study that looked at the effect of PML knock-down on HCMV infection showed that IE gene expression was elevated in these cells (22). In these cells, ND10 bodies are absent due to the degradation of PML, and thus, hDaxx remained diffuse throughout the cells. Although these data show a role for PML in the regulation of IE gene expression, they also suggest that the availability of co-factors and localization of hDaxx may be important for determining its repressive function on the MIEP.

Our experiments also indicated that repression of HCMV IE gene expression by stable overexpression of hDaxx could be overcome by increasing HCMV titers. Whether this titration of Daxx-mediated inhibition by increasing virus titers is mediated by viral structural proteins or simply increasing copy number of viral genomes will require further investigation. However, the pp71 tegument protein of HCMV, encoded by UL82, interacts with hDaxx (23), and this interaction is important for efficient IE gene expression and viral replication (25, 49, 50). Recently, it has also been reported that pp71 mediates the transient proteasomal degradation of hDaxx, leading to increased IE expression (26). The authors suggest that degradation of hDaxx by pp71 plays a profound role in regulation of viral IE gene expression. In this study, we show that cells stably overexpressing hDaxx are refractory to infection at an MOI of 1, a titer of virus that would normally result in 60% of cells infected. Since one con-

sequence of infection is the transient degradation of endogenous hDaxx between 3 and 12 h of infection (26), this suggests that, in cells overexpressing hDaxx or RFP-hDaxx, the incoming pp71 tegument protein is unable to overcome this stable overexpression of hDaxx. However, our observations that overexpressed hDaxx or RFP-hDaxx degradation does occur at higher MOIs suggest that this effect of overexpression hDaxx is not due to an intrinsic inability of overexpressed hDaxx to be degraded by pp71.

Our siRNA knock-down data also argue that the presence of hDaxx prior to the onset of its degradation by pp71, which starts at 2–3 h postinfection (26), as well as when it returns to wild-type levels at 12–24 h postinfection (49) still has profound effects on the level of IE gene expression despite the pp71-mediated transient degradation of endogenous hDaxx; specifically, we observe that even in the presence of pp71, inhibition of hDaxx expression prior to infection has profound effects on IE gene expression. Clearly, the presence of hDaxx in the cells within the first 2–3 h of infection, prior to its degradation by pp71, has an immediate effect at the chromatin level on the viral MIEP consistent with established interactions between hDaxx and repressive components of higher order chromatin structure formation (27). Indeed, we observed that the observed repression of the MIEP by hDaxx in these cells was alleviated by the addition of TSA, suggesting that chromatin remodeling enzymes are in an important factor in hDaxx repression of IE gene expression. Consequently, the knock-down of hDaxx prior to infection could result in the establishment of a chromatin structure around the MIEP that is more conducive to transcriptional activation prior to any delivery of pp71. Similarly, maintaining a knock-down of hDaxx expression by siRNA until later than 24 h postinfection (the time by which pp71-mediated degradation of hDaxx no longer occurs and hDaxx returns to wild-type levels) also profoundly increased IE gene expression in our study. Thus, it is likely that HCMV needs to employ multiple mechanisms over the immediate early time course of infection to overcome the intrinsic repression mediated by hDaxx; this probably also includes ND10 disruption by IE72 (6, 21, 22).

hDaxx then appears to act as an intrinsic inhibitor of efficient viral IE gene expression, which is mediated by packaging of the viral genome into a transcriptionally repressive chromatin state almost immediately upon infection, but this repressive function of hDaxx can be partially “overcome” by incoming viral factors, such as pp71, and is probably augmented by *de novo* expression of IE72 as the infection proceeds. During the course of this work, Preston and Nicholl (51) reported that siRNA knock-down of hDaxx results in increased IE expression after HCMV infection but only in the absence of incoming pp71 (using a pp71 deletion mutant). We are not sure why this discrepancy exists between our observations and theirs, but we note that, in contrast to our use of transiently siRNA transfected primary fibroblast cells, which virtually eliminated endogenous hDaxx (see Fig. 1A), they used G418^r-selected clones of immortalized U373 cells stably transfected with siRNA constructs in which endogenous hDaxx needed to be further reduced by a subsequent transfection protocol.

It would appear that the imposition of a transcriptionally inactive chromatin structure on the HCMV MIEP immediately

upon infection would be intrinsically unfavorable for the virus. However, herpesvirus gene expression is classically regulated in a temporal cascade, and this may be achieved, in part, using dynamic chromatin structure, a chromatin structure that is imparted on the incoming viral genome immediately upon infection. Similarly, if chromatinization of the viral MIEP into a more repressive structure occurs preferentially under cellular conditions that are suboptimal for replication, this may provide the virus an opportunity to synchronize its phase of IE gene expression to when the cellular conditions are more favorable. For example, cells that have progressed into late S phase do not appear to be conducive to viral IE expression, and viral replication does not occur until the cell cycle returns back into G₁/S phase, when the cell is believed to be an optimal environment for viral DNA replication (52). One possibility is that the virus utilizes chromatin structure to temporarily repress viral gene expression until the environment is more favorable for replication.

Given the dynamic nature of ND10 and ND10 components, it is likely that they may have a number of roles in infection, some supportive and others repressive. Most human herpesviruses have evolved mechanisms to disperse or destroy ND10 during the IE phase of the lytic cycle. A model in which ND10 functions in a repressive role prior to IE gene expression but in which their components then become beneficial to viral replication once released from the ND10 structure might explain such apparent contradictions in the literature. Indeed, it has been noted that infection of ND10-deficient cells with HCMV promotes the formation of rudimentary ND10-like structures of hDaxx and Sp100 (22). Further experimental approaches that critically examine specific ND10-virus interactions are required to clarify their role in virus infection.

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